REMARKS

In light of the Examiner's restriction and the current amendments, claims 111 and 114 are cancelled with the proviso that the subject matter may be later claimed by continuation or divisional application. Claims 94-113, 115 and 116 remain pending (claims 1-93 were previously cancelled).

Applicants appreciate the Examiner's comments regarding the objection to claims 94 and 108, and have amended the claims accordingly. In light of those amendments, claims 103 and 104 have also been simplified.

No new matter has been added.

Response under 35 U.S.C. §112, first paragraph.

The Examiner has rejected claims 101 and 115-116 under 35 U.S.C. §112, first paragraph, under the written description requirement regarding Applicants' use of the term "precursor prostaglandins." In making this rejection, the Examiner appears to be questioning whether, under the written description requirement, whether Applicants have presented any prostaglandin precursor other than latanoprost. See Applicants' specification at page 3, lines 27-28. This is not an issue of enablement, but rather one of written description.

It is well settled that while the purpose of §112, first paragraph, is to ensure that there is an adequate disclosure of the invention for which patent rights are sought and the purpose of the description requirement is to state what is needed to fulfill the enablement criteria, these requirements may be viewed separately but they are intertwined. *Kennecott Corp. v. Kyocera International, Inc.*, 5 USPQ2d 1194, 1197 (Fed. Cir. 1987) ("The written description must communicate that which is needed to enable the skilled artisan to make and use the claimed invention.")

Turning to the statement from Applicants' specification quoting the use of a prostaglandin precursor, refers to latanoprost in paragraph [0016] as "another new type of drug . . . which enhance outflow are also in current use." One of ordinary skill in the art would therefore, as part of his/her full knowledge, be familiar with such drugs if they are in current use, and would know which drugs are being referred to as a prostaglandin precursor for the stated purpose. Applicants have extended the definition of which drugs are intended by exemplifying

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latanoprost. Nowhere, however, do Applicants limit the claimed pharmaceutical composition to only latanoprost, nor are they so required by the law.

The courts have explained that "adequate description under the first paragraph of 35 U.S.C. §112 does not require literal support for the claimed invention. Rather, it is sufficient if the originally-filed disclosure would have conveyed to one of ordinary skill in the art that the appellant had possession of the concept of what is claimed." *Ex parte Parks*, 30 USPQ2d 1234, 1236 (BPAI 1993). Since the purpose of the law is to provide satisfaction of the description requirement to insure that subject matter presented in the form of a claim subsequent to the filing date of the application was sufficiently disclosed at the time of filing so that the prima facie date of invention can fairly be held to be the filing date of the application, Applicants have met this goal by not only providing the class of compositions referred to, but also an example of a member of the class known to the skilled practitioner..

> It is well established that a patent applicant is entitled to claim his invention generically, when he describes it sufficiently to meet the requirements of Section 112. "A specification may, within the meaning 112, first paragraph, contain a written description of a broadlyof 35 U.S.C. claimed invention without describing all species that claim encompasses." *Utter v. Hiraga* 6 USPQ2d 1709, 1714 (Fed. Cir. 1988). "Representative samples are not required by the statute and are not an end in themselves." *In re Robbins*, 166 USPQ 552, 555 (CCPA 1970). *Amgen, Inc. v. Chugai Pharmaceutical Co., Ltd.*, 18 USPQ2d 1016, 1027 (Fed. Cir. 1991).

As further explained by the court in *In re Metcalfe*, 161 USPQ 789 (CCPA 1969), "under appropriate circumstances an applicant may describe a material used in a claimed invention by referencing materials sold under a particular trade name or trademark." In this case, Applicants have exemplified one of a class of prostaglandins precursor drugs "currently in use," by naming latanoprost. Thus, Applicants have met the written description requirement by identifying the prostaglandins precursor drugs as those which would be readily recognized by the intended practitioner.

The Examiner also questions Applicants use of the term "cyclamate" as referenced in claims 115 and 116 (*i.e.*, the method of claim 108, wherein an anion comprising cyclamate is transferred into the ciliary epithelial cells of the aqueous humor to block native chloride channels). The Examiner says that the "cyclamate" referenced in claim 116 can only come from IB-MECA as shown in Figure 7, and that because claim 116 depends on claim 108, and claim 108 refers only

to treatment with an NHE inhibitor, that there is no support for the "cyclamate" because "there is no indication that IB-MECA is an NHE inhibitor.

In response to this conclusion, Applicants point out that, as claimed, IB-MECA is not claimed to be an NHE blocker. IB-MECA is a selective agonist (activator) of A3 adenosine receptors, which in turn activate chloride channels of nonpigmented (NPE) ciliary epithelial cells. Applicants have found that the cyclamate anion blocks the chloride channels that are believed to be important in aqueous humor formation. In short, Applicants' claimed invention addresses two different strategies: (i) use an antagonist to reduce chloride channel activity, and (ii) apply cyclamate to block whatever chloride channels remain open. Moreover, the cyclamate in Figure 7 was presented as one of several different anions to replace chloride, thereby characterizing the selectivity of the chloride channel. Thus, the Examiner is asked that this rejection of claims 115 and 116 be reconsidered and removed.

Response under 35 U.S.C. §112, second paragraph.

The Examiner has rejected claim 112 under 35 U.S.C. §112, second paragraph, as indefinite regarding Applicants' use of the term "analog." In making this rejection, the Examiner has kindly suggested that Applicants could substitute the word "derivative," which they have done by amendment, making this rejection moot. Applicants, therefore, ask that the Examiner reconsider and withdraw this rejection under §112, second paragraph. Note that claims 96 and 107 are similarly amended.

Response to Rejections under 35 U.S.C. §102(b).

Re: Cherksey

The Examiner has rejected claims 94-96, 102 and 105-107 under 35 U.S.C. §102(b) as anticipated by Cherksey (US Patent No. 4,950,591). In making this rejection, the Examiner relies upon Cherksey for the teaching that amiloride is an agent that blocks ion transport and interacts with a sodium-hydrogen exchange inhibitor and that amiloride and amiloride derivatives are capable of regulating membrane transport, cellular volume and other cellular pressure disorders. See columns 1-3.

However, in response to the Examiner's conclusions, Applicants point out that Cherksey's patent is actually very narrow, dealing with a peptide isolated from normal membranes that he isolated and found to conform to amiloride-sensitive channels. Amiloride

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blocks the sodium channel well. In fact, Merck developed that drug for the purpose of blocking the sodium channel. As a result, Cherksey's claims are solely for the use of the isolated peptide as a diagnostic and experimental tool, whereas by comparison, Applicants' invention neither teaches, nor claims, a method for regulating the sodium channel or its role in aqueous humor formation.

It must be recognized that Cherksey discovered neither sodium channels, nor their sensitivity to amiloride. For example, Cherksey refers to the review of Palmer (who received his doctorate degree under Dr. Civan), on the subject of amiloride-sensitive sodium channels. In fact, amiloride was used as a blocker in the first demonstration that it blocked a sodium channel (Lindemann *et al.*, "Sodium-specific membrane channels of frog skin are pores: current fluctuations reveal high turnover," *Science* 21, 195(4275):292-4 (1977)). In the prior art, Dr. Civan reported that his ". . . results indicated that epithelial Na+ channels with a high affinity to amiloride likely contribute to reabsorption of solute from the aqueous humor" and that the "epithelial Na^+ channel is activated by shrinkage and contributes to unidirectional reabsorption as aqueous humor" (see, Abstract by Civan *et al.*, "Potential contribution of epithelial Na^+ channel to net secretion of aqueous humor," *J. Exp. Zool.*, 279:498-503 (1997)) (copy attached). This was the *first* evidence that amiloride-sensitive sodium channels were likely present in the nonpigmented (NPE) ciliary epithelial cells. See also the concluding sentence of Civan *et al.*, 1997, wherein it is explained that ". . . future approaches to the medical treatment of glaucoma could well focus on increasing the rate of unidirectional reabsorption in order to reduce net aqueous flow." In other words, the sodium channels (including amiloride-sensitive NPE sodium channels) underlie *reabsorption* of aqueous humor fluid, thus reducing the net rate of aqueous humor formation (net inflow).

Accordingly, the prior art teaches that blocking the sodium channel with amiloride *increases* inflow, *resulting in increased intraocular pressure* – which is contrary to the clinical intent of Applicants' invention. On the basis of that information, a knowledgeable practitioner would be led to *stimulate* the NPE sodium channels; not block them.

Applicants' claims are directed to inhibiting "sodium-hydrogen antiport activity" by administering to ciliary epithelial cells a pharmaceutical composition comprising or consisting essentially of a pressure-modulating amount of at least one "sodium-hydrogen exchange (NHE) inhibitor." Thus, Cherksey not only fails to anticipate Applicants' invention, it actually leads

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one away from what is taught by Applicants' patent application regarding regulation of the antiports. It also teaches away from what has been demonstrated in the prior art by Avila *et al.*, "Inhibitors of NHE-1 Na+/H+ exchange reduce mouse intraocular pressure," *Invest. Ophthalmol. Vis. Sci.* 43:1897-1902 (2002)) (demonstrating that the topical application of NHE inhibitors lowers IOP). Copy attached.

Moreover, Cherksey's speculation that amiloride-sensitive channels could be inhibited in dealing with many diseases, including glaucoma is unsupported and not enabled by the cited patent which teaches only methods relating to sodium channels. No evidence, let alone a rationale basis, is provided in the cited patent or anywhere in the prior art for such speculation, and others could not be taught by a mere speculation. More importantly, Cherksey neither mentions, nor suggests, that inhibiting or blocking NHE exchange would reduce aqueous humor inflow or intraocular pressure. Thus, the method taught by Cherksey fails to address each and every element of Applicants' claimed invention, and as such, the reference fails to anticipate the present invention.

Accordingly, Applicants respectfully request that the rejection of claims 94-96, 102 and 105-107 under 35 U.S.C. §102(b) be reconsidered and withdrawn. Note that the cited references are provided to discuss the sodium channels raised by the Examiner, not as prior art. Thus, although attached, the references are not identified in a Supplemental IDS with the necessary fees. However, should the Office require submission of an IDS, and notifies Applicants' representative, Applicants will so file.

Re: "Drug Facts and Comparisons"

The Examiner has also rejected claims 94 and 102-105 under 35 U.S.C. §102(b) as being unpatentable "Drug Facts and Comparisons" (1994). In making this rejection, the Examiner relies upon "Drug Facts and Comparisons" for teaching the use of timolol, which the Examiner defines as a beta blocker in reliance on the prior art and on Applicants' list at page 6, lines 23-26. However, as previously shown on the record, by cited prior art and by Declaration in Applicants' prior Response dated November 10, 2005, timolol was not, recognized by those knowledgeable in the field to be a sodium-hydrogen exchange (NHE) inhibitor.

Regardless of the Examiner's arguments that reduction of intraocular pressure is demonstrated by the use of timolol in "Drug Facts and Comparisons," the reference offers no evidence that timolol achieved any inhibition of sodium-hydrogen antiport activity *in the ciliary*

epithelial cells. It describes on a change in intraocular pressure, not any effect what-so-ever on the ciliary epithelial cells. In fact, the effect of any pharmaceutical composition on the antiports and the effect of such treatment was unknown until its discovery by the present inventors, so it could not have been known or suggested by the art. As a result, nowhere in the prior art is there a suggestion that the antiports controlled fluid build up in the aqueous humor, and nowhere is there a suggestion that the NHE inhibitors could control the activity of the antiports. Yet, that is what is claimed by Applicants - not simply a possible effect on intraocular pressure.

Applicants have added to the previously claimed step of "administering" of the pharmaceutical composition in their claimed method, a second step that expressly requires" inhibiting sodium-hydrogen antiport activity" in the ciliary epithelial cells. This further emphasizes that, while "Drug Facts and Comparisons" may say that a small reduction of intraocular pressure was noted in the subject animal, the cited art does not offer any treatments of antiport activity – yet regulating antiport activity is expressly Applicant's invention – not simply reducing intraocular pressure. Thus, there is no need to read subject matter from the specification into the claims. It is now clearly stated.

This is not a case of inherency because while "Drug Facts and Comparisons" may report a reduction of intraocular pressure in the subject animal in conjunction with treatment, there is no way of knowing that that reduction was *a result of inhibition or regulation of antiport activity.* The Examiner asks on page 8 how Applicants can "bypass administering an eyedrop to the eye to administer said compositions to the ciliary epithelial cells of the aqueous humor." But then the Examiner turns to *Continental Can* to say that "a prior art reference may anticipate without disclosing a feature of the claimed invention, if that missing characteristic is necessarily present or inherent, in the single anticipating reference." How are these two concepts connected? Where is it described exactly how the change in intraocular pressure was tested by the authors of "Drug Facts and Comparisons" after what appears to be topical application?

In citing *Continental Can*, the Office must look at the entire holding of the decision, which further explains that

> To serve as an anticipation when the reference is silent about the asserted inherent characteristic, such gap in the reference may be filled with recourse to extrinsic evidence. Such evidence must make clear that the missing descriptive matter is necessarily present in the thing described in the reference, and that it would be so recognized by persons of ordinary skill. *Continental Can Co. USA Inc. v. Monsanto,* 20 USPQ2d 1746, 1749 (Fed. Cir. 1991).

It cannot be assumed, without some evidence supporting the conclusion that the treatment by the authors of "Drug Facts and Comparisons" "could" have regulated or altered the antiport activity, or that such action on the antiports was inherent based upon the limited disclosure in the cited reference. If one were to use that definition, all things are inherent, because if you add enough links to what the inventors now disclose, there is nothing new under the sun. That of course, is not the basis for patent law; rather "new" discoveries are patentable.

It would appear that only Applicants' own invention has led to the conclusion that the treatment described in "Drug Facts and Comparisons" anticipates (inherently or otherwise) Applicants' claimed invention that regulates antiport activity. This is not a permissible basis for rejection. Applicants' claims are anticipated "only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference." *Verdegaal Brothers, Inc. v. Union Oil Co. of Calif.*, 2 USPQ2d 1051, 1053 (Fed. Cir. 1987). "Inherency may not be established by probabilities or possibilities. The mere fact that a certain thing may result from a given set of circumstances is not sufficient." *Ex parte Skinner*, 2 USPQ2d 1788, 1789 (BPAI 1987).

Accordingly, Applicants' respectfully ask that in the accompanying RCE, that the invention be reconsidered be withdrawn.

Response to Rejections under 35 U.S.C. §103(a).

Re: Adorante and Cherksey.

The Examiner has rejected claims 94-96, and 99-113 under 35 U.S.C. §103(a) as unpatentable over Adorante (US Patent No. 5,559,151) and Cherksey (US Patent No. 4,950,591). In making this rejection, the Examiner relies on Adorante for the use of 4,4'-diisothiocyanato-stilbene-2,2'-disulfonate (DIDS) to treat glaucoma and/or ocular hypertension, although the Examiner agrees that Adorante fails to suggest co-administration of NHE/NHE1 inhibitors. However, the Examiner further combines Cherksey with Adorante for the teaching that amiloride blocks ion transport and interacts with a sodium-hydrogen exchange inhibitor and that amiloride and amiloride derivatives are capable of regulating membrane transport, cellular volume and other cellular pressure disorders. The Examiner's conclusion is based on the premise that it would have been obvious to "employ two agents well known to treat glaucoma/ocular hypertension together to treat the very same condition.

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In response, Adorante actually proposes the use of DIDS as a chloride-channel blocker of NPE cells, without any reference what- so-ever to bicarbonate-chloride exchange. This offers no relevance to blocking of sodium-proton exchange, and even the Examiner asserts that Adorante fails to suggest administration of NHE/NHE1 inhibitors. Therefore, alone, Adorante has no effect on the patentability of Applicants' invention.

However, in this rejection, Adorante is not cited alone. It is combined with Cherksey. But, for the above stated reasons, Cherksey teaches a method for treating intraocular pressure by administering to affect the *sodium channels* of the eye, but that is not Applicants' invention, nor do Applicants' address the role of the sodium channel in aqueous humor formation. In fact, as explained above, if applied to Applicants' claimed invention, Cherksey would actually teach away from the invention. In addition, if the Office is tempted to find that the use of amiloride inherently teaches Applicants' invention, they are directed to *Jones v. Hardy* 220 USPQ 1020, 1025 (Fed. Cir. 1984) (The fact that a claimed invention is based on an inherent quality of a product well known in the art does not mean the invention is obvious as this confuses anticipation by inherency with obviousness).

Consequently, linking DIDS that blocks chloride channels (Adorante) with amiloride that blocks the ENaC sodium channel (Cherksey) would in no way lead one of skill in the art to try blocking sodium-proton exchange in order to reduce inflow at the antiports at the time of Applicants' invention. Even if combined, the combination fails to teach each and every element of Applicants' claimed invention. Since Cherksey fails to teach administering NHE/NHE1 inhibitors to the antiports, it cannot supplement the gap left by Adorante, and Adorante cannot render Applicants' invention obvious. Accordingly, Applicants respectfully request that the rejection of claims 94-96, and 99-113 under 35 U.S.C. §103(a) be reconsidered and withdrawn.

Re: Brandt and Cherksey.

The Examiner has rejected claims 94-98, and 102-113 under 35 U.S.C. §103(a) as unpatentable over Brandt (US Patent No. 5,559,151) and Cherksey (US Patent No. 4,950,591). In making this rejection, the Examiner relies on Brandt for the use of an inhibitor of a Na^+-K^+-$2Cl^-$ (symport), such as butetanide, to treat glaucoma and/or ocular hypertension, although the Examiner agrees that Brandt fails to suggest co-administration of NHE/NHE1 inhibitors. However, the Examiner further combines Cherksey with Brandt for the teaching that amiloride blocks ion transport and interacts with a sodium-hydrogen exchange inhibitor and that amiloride

and amiloride derivatives are capable of regulating membrane transport, cellular volume and other cellular pressure disorders. The Examiner's conclusion is based on the premise that it would have been obvious to "employ two agents well known to treat glaucoma/ocular hypertension together to treat the very same condition.

In response, even if Brandt proposed the use of an inhibitor of a $Na^+-K^+-2Cl^-$ (symport), such as bumetanide, it is irrelevant to Applicants' invention. This is because Dr. Civan and others have demonstrated that bumetanide is, by itself, ineffective in lowering IOP. See, attached, Tian *et al.* "Effects of Adenosine Agonists on Intraocular Pressure and Aqueous Humor Dynamics in Cynomolgus Monkeys," *Exp. Eye Res.* 64:979-989 (1997) (demonstrating that bumetanide had no effect on IOP of live monkeys). Subsequently, Dr. Civan and associates demonstrated that bumetanide also has no effect on IOP of the live mouse, and it lowers IOP only if the sodium-proton exchange is also blocked (see, attached 2002 Avila *et al.*, reference in *Invest. Ophthalmol. Vis. Sci.* 43:1897-1902). Consequently, in light of the prior art at the time, Brandt's patent teaches that blocking the sodium-potassium-chloride co-transporter was the controlling factor in the process, not inhibition of sodium-proton exchange. Thus, Brandt has no relevance to blocking of sodium-proton exchange, and even the Examiner asserts that Brandt fails to suggest administration of NHE/NHE1 inhibitors. Therefore, alone, Brandt has no effect on the patentability of Applicants' invention.

However, in this rejection, Brandt is not cited alone. It is combined with Cherksey. But, for the above stated reasons, Cherksey teaches a method for treating intraocular pressure by administering to affect the amiloride-sensitive sodium channel, but that is not Applicants' invention, nor do Applicants' address the role of the sodium channel in aqueous humor formation. In fact, as explained above, if applied to Applicants' claimed invention, Cherksey would actually teach away from the invention.

Consequently, linking treatment of bumetanide as an inhibitor of a $Na^+-K^+-2Cl^-$ (symport) (Brandt) with amiloride that blocks the ENaC sodium channel (Cherksey) would in no way lead one of skill in the art to try blocking sodium-proton exchange in order to reduce inflow at the antiports at the time of Applicants' invention. Even if combined, the combination fails to teach each and every element of Applicants' claimed invention. In addition, if the Office is tempted to find that the use of amiloride inherently teaches Applicants' invention, they are directed to *Jones v. Hardy* 220 USPQ 1020, 1025 (Fed. Cir. 1984) (The fact that a claimed

invention is based on an inherent quality of a product well known in the art does not mean the invention is obvious as this confuses anticipation by inherency with obviousness).

Since Cherksey fails to teach administering NHE/NHE1 inhibitors to the antiports, it cannot supplement the gap left by Brandt, and Brandt cannot render Applicants' invention obvious. Accordingly, Applicants respectfully request that the rejection of claims 94-98, and 102-113 under 35 U.S.C. §103(a) be reconsidered and withdrawn.

Note that the Examiner cites Shell (US Patent No. 4,281,654) on form 892, but no citation to the reference can be found in the Office Action, so it is presumed to be cited for record purposes only.

Barring other rejections, therefore, Applicants submit that their pending claims are patentable, and ask that the present rejections be entirely overturned and withdrawn. Please contact Applicants' undersigned representative at (215) 772-7550 if further discussion of this case is needed. This response is filed without extension fess, but with the fee for an RCE. If there are any additional fees due in connection with the filing of this response, please charge the fees to our Deposit Account No. 50-2424.

Respectfully submitted,

Date: <u>October 31, 2007</u>

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Potential Contribution of Epithelial Na+ Channel to Net Secretion of Aqueous Humor

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ABSTRACT   The aqueous humor of the eye is secreted by the bilayered ciliary epithelium, consisting of the pigmented (PE) cell layer facing the stroma and the nonpigmented (NPE) cell layer facing the aqueous humor. Cells within each layer and between the two layers are linked by gap junctions, forming a ciliary epithelial syncytium. Unidirectional secretion from the stroma to the aqueous proceeds both through the cells (the transcellular pathway) and between the cells (the paracellular pathway). Net formation of aqueous humor must, however, be the algebraic sum of unidirectional secretion and unidirectional reabsorption from the aqueous humor back into the stoma. The mechanisms potentially underlying reabsorption of aqueous humor by the NPE cells have recently been addressed by studying the regulatory response (RVI) of anisosmotically shrunken NPE cells. The results indicated that epithelial Na+ channels with a high affinity to amiloride likely contribute to reabsorption of solute from the aqueous humor. We have substantiated this possibility by using Northern analysis to identify in human ciliary body RNA a 3.7-kb transcript corresponding to the α-subunit of the amiloride-sensitive, αβγ-ENaC epithelial sodium channel. We have also found that the Na+-channel inhibitor benzamil inhibits the RVI without affecting the cell volume of isotonic cell suspensions. This observation supports the hypothesis that the low conductance, highly selective epithelial Na+ channel is activated by shrinkage and contributes to unidirectional reabsorption as aqueous humor. Examples are provided of how the integrative regulation of aqueous humor formation can involve conjugate actions on both unidirectional secretion and reabsorption. *J. Exp. Zool. 279:498–503, 1997.*   © 1997 Wiley-Liss, Inc.

STRUCTURE OF THE CILIARY EPITHELIUM

The aqueous humor of the eye is secreted by the ciliary epithelium, a bilayered structure covering the surface of the ciliary body. The outer cell layer is formed by the pigmented ciliary epithelial (PE) cells, whose basolateral membranes face the ciliary body stroma. The inner cell layer consists of the nonpigmented ciliary epithelial (NPE) cells, whose basolateral surfaces face the aqueous humor. Only the NPE cells form tight junctions in vivo (Raviola and Raviola, '78). Low-resistance gap junctions connect cells within each layer and between the two layers, creating a functional syncytium (Raviola and Raviola, '78; Green et al., '85; Coca-Prados et al., '92; Edelman et al., '94; Oh et al., '94; Bowler et al., '96).

OVERVIEW OF CILIARY EPITHELIAL SECRETION

As in the case of transepithelial transport generally, fluid is translocated by the primary transfer of solute from the stroma to the aqueous humor, with water passively following. The slightly negative transepithelial potential (of ~1 mV) provides a driving force for Na+ and other cations to move through the paracellular pathway (between the cells). However, the dominant contribution to unidirectional secretion of solute is likely to proceed by the transcellular pathway (through the cellular syncytium; Krupin and Civan, '95). Transcellular

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transfer from the stroma to the contralateral surface must involve at least three steps, each of which could theoretically be the rate-limiting process for aqueous humor formation. First, solute is taken up from the stroma by the PE cells; this step has been thought to be largely performed by a Na^+-K^+-$2Cl^-$ symport (Krupin and Civan, '95), but possibly also by Cl^-/HCO_3^- antiport exchange (Wiederholt et al., '91). Second, solute and water diffuse from the PE to the NPE cells through the gap junctions. Third, Na^+, K^+, and Cl^- are released by the NPE cells into the aqueous humor through the Na^+/K^+ exchange pump, K^+ channels, and Cl^- channels, respectively.

Recent results obtained by electron probe X-ray microanalysis of intact rabbit ciliary epithelium (Bowler et al., '96) bear on the issue of the rate-limiting site of aqueous humor formation. As summarized in Table 1, the elemental Cl concentration (in mmol/kg intracellular water) is some four-to-five times higher in the PE cells than the equilibrium distribution predicted at a membrane potential of $\sim$–70 mV (Carré et al., '92). This datum indicates that the PE cells can accumulate solute against an electrochemical gradient under baseline conditions. The data also document that the intracellular elemental concentrations of Na, K, and Cl are approximately equal in the PE and NPE cells. This observation indicates that the gap junctions are also unlikely to limit the flow of solute from stroma to aqueous humor; otherwise, we would expect to find gradients in concentration between the PE and NPE cells. By exclusion, we conclude that the third step, the final net release of solute from the NPE cells into the aqueous humor is likely to limit the rate of ciliary epithelial secretion.

The net translocation of solute from the NPE cells into the aqueous must equal the difference between unidirectional secretion out of the cells and unidirectional reabsorption back into the cells. These two unidirectional fluxes appear to reflect the operation of very different transport mecha-

TABLE 1. *Intracellular composition of intact rabbit ciliary epithelium*[1]

| Component | Concentration (mmol/kg intracellular water) | | |
	PE cells	NPE cells	All cells
Na	13 ± 2	16 ± 2	15 ± 3
K	164 ± 12	162 ± 8	162 ± 14
Cl	44 ± 3	48 ± 3	46 ± 5

[1]The entries are the means ± SE taken under baseline conditions from the electron probe X-ray microanalyses of Bowler et al. ('96).

nisms. In contrast to the Na^+/K^+ exchange pump, K^+ channels and Cl^- channels subserving unidirectional secretion, four different mechanisms (considered below) seem to underlie unidirectional reabsorption. Recent volumetric measurements of human NPE cells have suggested that one of the mechanisms underlying unidirectional reabsorption is an amiloride-sensitive epithelial Na^+ channel (Civan et al.,'96). These measurements were conducted with the simplest possible experimental model of aqueous humor reabsorption, the regulatory volume increase (RVI) of NPE cells (Civan et al.,'96). The human (ODM) NPE cells are first suspended in 50% hypotonic solution, producing swelling and a secondary release of KCl and water. Isotonicity is then restored by addition of sucrose 25 min after the initial suspension. The cells shrink to $\sim$80% of their isotonic volumes, but then exhibit a secondary regulatory response (the RVI) in which solute and water are slowly taken up at 0.144 ± 0.007%/min (Civan et al.,'96). The RVI was significantly inhibited by amiloride at 20 μM, dimethylamiloride at 10 μM, and benzamil at 1 μM; the effects of amiloride and dimethylamiloride were not significant at 1 μM (Civan et al.,'96). Benzamil is more effective than amiloride in blocking high amiloride-affinity epithelial Na^+ channels, whereas dimethylamiloride is more effective than amiloride in blocking Na^+/H^+ antiport exchange (Kleyman and Cragoe, '88). These results were consistent with the pharmacologic profile characterizing low amiloride-affinity Na^+ channels (Oh and Benos,'92). However, an alternative interpretation was possible. The human NPE cells also display Na^+/H^+ antiport activity (Civan et al., '96). Thus, the observed inhibitions of the RVI could have reflected the actions of amiloride and its analogues on two different transport targets: benzamil could have been blocking high amiloride-affinity Na^+ channels [to which the $\alpha\beta\gamma$-ENaC heterotrimeric channel (Canessa et al., '94) belongs] and dimethylamiloride could have been acting solely on the Na^+/H^+ antiport. Thus, the data indicated the operation of a Na^+ channel, but did not resolve whether the channel conformed to a high- or low-affinity class Na^+ channels, so that its molecular identity was unknown.

IDENTIFICATION AND REGULATION OF AN EPITHELIAL NA$^+$ CHANNEL

In the present manuscript, we report finding the expression of an amiloride-sensitive Na^+ channel in the human ciliary body. Total RNA (20 μg) was

extracted (Chomczynski and Sacchi, '87) from the ciliary body of a human eye and separated in a 1%-agarose gel, blotted and hybridized in high-stringency conditions (Coca-Prados et al., '96) with a probe for the expression of transcripts of the $\alpha\beta\gamma$-ENaC heterotrimeric, amiloride-sensitive channel (Canessa et al., '94). The probe chosen was a 1.9-kb DNA probe corresponding to an EcoRI/BamHI fragment of the α-subunit of the rat amiloride-sensitive epithelial sodium channel (a gift of Dr. Cecilia M. Canessa, Yale University).

Figure 1 presents a resulting Northern blot analysis. At the left are the migration positions of standard RNA molecular markers. The arrow at the right indicates the position of the major 3.7-kb transcript specific for the α-subunit mRNA. It is likely that this probe of the $\alpha\beta\gamma$-ENaC epithelial Na$^+$ channel has detected mRNA from the epithelial cells. However, it should be noted that

other cells of the human ciliary body (such as smooth muscle) may also have contributed to the transcript detected.

The results of Figure 1 support the concept that the $\alpha\beta\gamma$-ENaC epithelial Na$^+$ channel supports reabsorption of Na$^+$ from the aqueous humor back into the NPE cells. However, it was unclear whether this Na$^+$-channel activity was expressed under baseline conditions or only after stimulation (in this case, by shrinkage). Recently, we have measured the effects of 1 μM benzamil on parallel aliquots of isotonic suspensions of human NPE cells before and after exposure to 50%-hypotonic solutions, following the approach of Civan et al. ('96). Benzamil had no detectable effect on the baseline isotonic volume at the same concentration which inhibited the RVI (Fig. 2). We conclude that shrinkage activates epithelial Na$^+$ channels of human NPE cells, as has been noted for other epithelial cells (Okada and Hazama, '88; Wehner et al., '95).

COMPONENTS UNDERLYING VECTORIAL TRANSPORT AT THE NPE-AQUEOUS HUMOR INTERFACE

The foregoing results indicate shrinkage-activated $\alpha\beta\gamma$-ENaC epithelial Na$^+$ channels participate in reabsorption of aqueous humor back into the NPE cells. Volumetric measurements of immortalized human (ODM) NPE cells indicate that three other mechanisms can also underlie unidirectional reabsorption: a Na$^+$/H$^+$ antiport in parallel with a Cl$^-$/HCO$_3^-$ antiport, a diazide-sensitive Na$^+$-Cl$^-$ symport, and the bumetanide-sensitive Na$^+$-K$^+$-2Cl$^-$ symport (Civan et al., '96).

As noted above, unidirectional secretion at the basolateral surface of the NPE cells is likely to proceed through the Na$^+$/K$^+$ exchange pump and through parallel K$^+$ and Cl$^-$ channels. Different isozymes of the pump appear to be operative in the pars plana and pars pliccata areas of the ciliary epithelium (Ghosh et al., '90). The precise molecular identities of the K$^+$ channels have not yet been identified, but inward rectifiers, delayed rectifiers and calcium-activated outward rectifiers have been observed with NPE cells (Jacob and Civan, '96). The identity of the Cl$^-$ channels has not yet been unequivocally established. On the basis of their results, Coca-Prados et al. ('96) have suggested that the volume-sensitive protein pI$_{Cln}$ may be regulating a conduit formed by the protein kinase C (PKC)-inhibitable ClC-3 Cl$^-$ channel in human NPE cells. Results obtained with an antibody of P-glycoprotein suggest that this
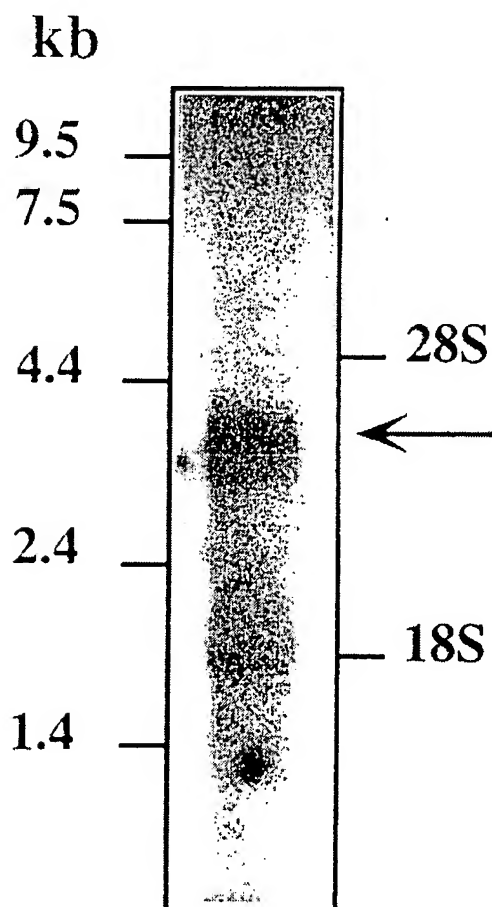

Fig. 1. Expression of an amiloride-sensitive Na$^+$ channel in the human ciliary body. At left are the migration positions of standard RNA molecular markers. The arrow at the right indicates the position of the major 3.7-kb transcript specific for the α-subunit mRNA.
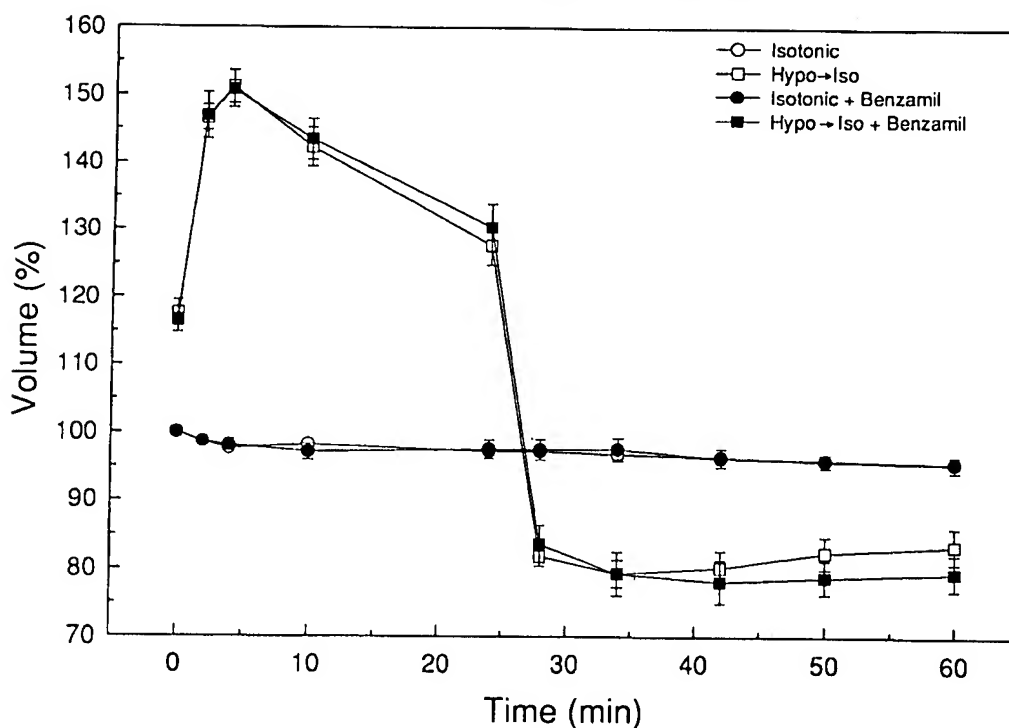
Fig. 2. Effects of benzamil on the regulatory volume increase (RVI) and cell volume in isosmotic suspensions. The cell volumes were measured by electronic cell sorting (Civan et al., '96) in the absence (open symbols) or presence (closed symbols) of 1 μM benzamil. The cell volumes have been normalized to the initial value in isotonic medium. The square symbols present data points (±1 SE) for human NPE (ODM) cells suspended in hypotonic Ringer's solution containing (in mM): 55.0 NaCl, 7.5 HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], 1.2 $CaCl_2$, 0.6 $MgCl_2$, 2.4 KCl, 0.6 KH_2PO_4, 15 $NaHCO_3$, and 10.0 glucose. In the absence of added sucrose, the external osmolality was hypotonic (150–160 mOsm), producing swelling and triggering a secondary regulatory reduction in volume over the 4–24 min following suspension. Sufficient sucrose was added 25 min after suspension to bring the osmolality to isotonicity (300–315 mOsm), thereby shrinking the cells until the appearance of a regulatory volume increase (RVI) over the period t = 34–60 min. Measured by linear least-square regression, the rate of swelling during the RVI displayed by the control aliquots was 0.162 ± 0.024%/min (n = 8). Benzamil reduced the rate of the RVI in paired aliquots to 0.012 ± 0.036%/min (P < 0.025 by Student's paired t-test). In contrast, the same concentration of benzamil had no detectable effect on the cell volume of aliquots suspended in isotonic solution (n = 4). The four isotonic experiments were conducted in parallel with four of the eight RVI experiments and have not been previously reported. The additional four sets of measurements included with the RVI experiments were obtained from the results of Civan et al. ('96).

protein product of MDR1 may also regulate Cl⁻ currents in bovine NPE cells (Wu et al., '96).

CONJUGATE REGULATION OF UNIDIRECTIONAL SECRETION AND REABSORPTION

With rare exception (Sears, '84), studies of net ciliary epithelial secretion have largely focused on the unidirectional transfer of solutes and water from the stroma to the aqueous humor. However, in principle, we might expect that secretagogues could well have complementary effects on unidirectional secretion and reabsorption. Otherwise, stimulation or inhibition of both antiparallel flows might have no effect on net secretion. As noted above, shrinkage stimulates Na^+-channel activity of human NPE

cells. Since ciliary epithelial cells are unlikely to undergo great changes in volume in vivo, we have also examined the potential regulatory roles of other modifiers of net secretion. Inhibition of PKC with staurosporine activates Cl⁻ channels of human NPE cells (Civan et al., '94; Coca-Prados et al., '95, '96); and activation of PKC has the opposite effect (Civan et al., '94). In order to examine the effects of staurosporine on the reverse flow, we have again turned to the simplest possible experimental model of aqueous humor reabsorption, the regulatory volume increase (RVI) of NPE cells. In contrast to its stimulation of Cl⁻ secretion, staurosporine inhibits RVI (Civan et al., '96). These conjugate effects of inhibiting PKC activity are illustrated in the cartoon of Figure 3.
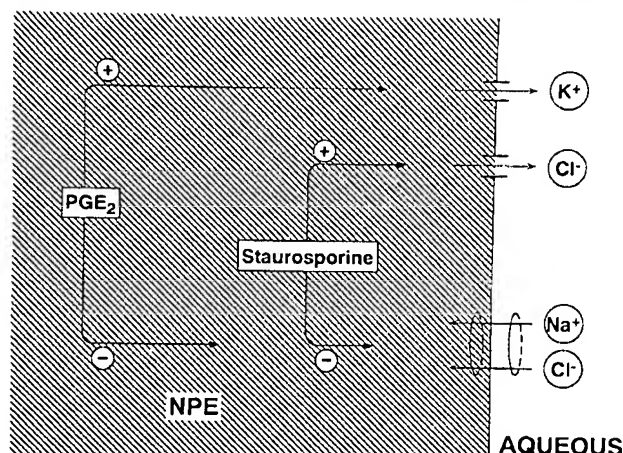
Fig. 3. Postulated regulation of net aqueous humor formation by conjugate modulation of unidirectional secretion and unidirectional reabsorption by the NPE ciliary epithelial cells.

The actions of the arachidonic acid metabolite PGE_2 provide a second, slightly more complex example of the conjugate modification of unidirectional secretion and reabsorption (Fig. 3). PGE_2 also stimulates Cl^- secretion, but its action is indirect, at least in human ODM NPE cells. The prostanoid increases K^+-channel activity (Civan et al., '94), hyperpolarizing the membrane, and thereby providing an increased electrical driving force for Cl^- release into the aqueous humor. Like staurosporine, PGE_2 inhibits the RVI of the NPE cells (Civan et al., '96).

To the extent that the RVI can be taken as an index of reabsorption of aqueous humor by the NPE cells, these results indicate that second messenger cascades can exert complementary actions on unidirectional secretion and reabsorption. Reductions in unidirectional reabsorption as well as increases in unidirectional secretion can enhance the net rate of aqueous humor formation. The converse is also possible, so that future approaches to the medical treatment of glaucoma could well focus on increasing the rate of unidirectional reabsorption in order to reduce net aqueous flow.

ACKNOWLEDGMENTS

LITERATURE CITED

Bowler, J.M., D. Peart, R.D. Purves, D.A. Carré, A.D.C. Macknight, and M.M. Civan (1996) Electron probe X-ray microanalysis of rabbit ciliary epithelium. Exp. Eye Res., 62:131–139.

Canessa, C.M., L. Schild, G. Buell, B. Thorens, I. Gautschi, J.-D. Horisberger, and B.C. Rossier (1994) Amiloride-sensitive epithelial Na^+ channels is made of three homologous subunits. Nature, 367:463–467.

Carré, D.A., C.-S.R. Tang, T. Krupin, and M.M. Civan (1992) Effect of bicarbonate on intracellular potential of rabbit ciliary epithelium. Curr. Eye Res., 11:609–624.

Chomczynski, P., and N. Sacchi (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., 162:156–159.

Civan, M.M., M. Coca-Prados, and K. Peterson-Yantorno (1994) Pathways signalling the regulatory volume decrease of cultured non-pigmented ciliary epithelial cells. Invest. Ophthalmol. Vis. Sci., 35:2876–2886.

Civan, M.M., M. Coca-Prados, and K. Peterson-Yantorno (1996) Regulatory volume increase of human non-pigmented ciliary epithelial cells. Exp. Eye Res., 62:627–640.

Coca-Prados, M., J. Anguíta, M.L. Chalfant, and M.M. Civan (1995) PKC-sensitive Cl^- channels associated with ciliary epithelial homologue of pI_{Cln}. Am. J. Physiol., 268:C572–C579.

Coca-Prados, M., S. Ghosh, N.B. Gigula, and N.M. Kumar (1992) Expression and cellular distribution of the α1-gap junction gene product in the ocular pigmented ciliary epithelium. Curr. Eye Res., 11:113–122.

Coca-Prados, M., J. Sánchez-Torres, K. Peterson-Yantorno, and M.M. Civan (1996) Association of CIC-3 channel with Cl^- transport by human nonpigmented ciliary epithelial cells. J. Membrane Biol., 150:197–208.

Edelman, J.L., G. Sachs, and J.S. Adorante (1994) Ion transport asymmetry and functional coupling in bovine pigmented and nonpigmented ciliary epithelial cells. Am. J. Physiol., 266:C1210–C1221.

Ghosh, S., A.C. Freitag, P. Martin-Vassallo, and M. Coca-Prados (1990) Cellular distribution and differential gene expression of the three α subunit isoforms of the Na^+, K^+-ATPase in the ocular ciliary epithelium. J. Biol. Chem., 265:2935–2940.

Green, K., C. Bountra, P. Georgiou, and C.R. House (1985) An electrophysiologic study of rabbit ciliary epithelial cells. Invest. Ophthalmol. Vis. Sci., 26:371–381.

Kleyman, T.R., and E.J. Cragoe, Jr. (1988) Amiloride and its analogs as tools in the study of ion transport. J. Membrane Biol., 105:1–21.

Krupin, T., and M.M. Civan (1995) The physiologic basis of aqueous humor formation. In: The Glaucomas. 2nd ed., Ritch, R., M.B. Shields, and T. Krupin, eds., C.V. Mosby, St. Louis, pp 251–280.

Oh, J., T. Krupin, L.Q. Tang, J. Sveen, and R.A. Lahlum (1994) Dye coupling of rabbit ciliary epithelial cells in vitro. Invest. Ophthalmol. Vis. Sci., 35:2509–2514.

Oh, Y.S., and D.J. Benos (1992) Amiloride-sensitive sodium channels. In: Amiloride and Its Analogs: Unique Cation Transport Inhibitors. Cragoe, E.J., Jr., T.R. Kleyman, and L. Simchowitz, eds., VCH, New York, pp 41–56.

Okada, Y., and A. Hazama (1989) Volume-regulatory ion channels in epithelial cells. NIPS 4:238–242.

Raviola, G., and E. Raviola (1978) Intercellular junctions in the ciliary epithelium. Invest. Ophthalmol. Vis. Sci., 17:958–981.

Sears, M.L. (1984) Autonomic nervous system: Adrenergic agonists. In: Pharmacology of the Eye, M.L. Sears, ed. Springer-Verlag: New York, pp 193–248.

Wehner, F., H. Sauer, and R.K.H. Kinne (1995) Hypertonic stress increases the Na$^+$ conductance of rat hepatocytes in primary culture. J. Gen. Physiol., *105*:507–535.

Wiederholt, M., H. Helbig, and C. Korbmacher (1991) Ion transport across the ciliary epithelium: Lessons from cultured cells and proposed role of the carbonic anhydrase. In: Carbonic Anhydrase. F. Botre, G. Gross, B. Storey, eds., VCH, Wernheim, Germany, pp 232–244.

Wu, J., J.J. Zhang, H. Koppel, and T.J.C. Jacob (1996) Evidence for an association between P-glycoprotein and volume-activated chloride currents in non-pigmented ciliary epithelial cells. J. Physiol., *491.3*:743–755.

Inhibitors of NHE-1 Na$^+$/H$^+$ Exchange Reduce Mouse Intraocular Pressure

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PURPOSE. To test whether blocking the Na$^+$/H$^+$ antiport reduces intraocular pressure (IOP) in the mouse.

METHODS. The electrophysiologic approach (the servo-null micropipette system, SNMS) that had been adapted for continuously monitoring IOP in the mouse was used in a study of the effects of a series of transport inhibitors.

RESULTS. Topical application of three direct blockers of Na$^+$/H$^+$ exchangers produced comparable reductions in mouse IOP: dimethylamiloride (DMA, -5.0 ± 0.7 mm Hg), ethylisopropylamiloride (EIPA, -4.1 ± 1.0), and BIIB723 (-4.9 ± 1.7 mm Hg). These effects were mediated locally, not systemically, because adding DMA to one eye had no effect on IOP in the contralateral eye. In contrast to the actions of selective inhibitors of Na$^+$/H$^+$ exchange, neither the low-potency inhibitor amiloride nor the inhibitor of Na$^+$-K$^+$-2Cl$^-$ cotransport bumetanide by itself was effective. Dorzolamide, which slows delivery of H$^+$ and HCO$_3^-$ to Na$^+$/H$^+$ and Cl$^-$/HCO$_3^-$ antiports, also reduced IOP by 2.9 ± 0.6 mm Hg. After first blocking Na$^+$/H$^+$ exchange with DMA, EIPA, BIIB723, or dorzolamide, application of bumetanide produced an additional reduction in IOP of 3.8 to 4.0 mm Hg.

CONCLUSIONS. The first step in formation of aqueous humor is uptake of NaCl by the ciliary epithelial cells from the stroma, possibly by both paired Na$^+$/H$^+$ and Cl$^-$/HCO$_3^-$ antiports and a bumetanide-sensitive Na$^+$-K$^+$-2Cl$^-$ symport. The present data are consistent with electron probe x-ray microanalyses of rabbit ciliary epithelium indicating that the antiports are the dominant mechanism. That bumetanide can produce a previously unobserved lowering of IOP when the Na$^+$/H$^+$ antiport is also inhibited substantiates a dominant antiport mechanism. *(Invest Ophthalmol Vis Sci.* 2002;43:1897–1902)

Intraocular pressure (IOP) reflects a balance between inflow across the ciliary epithelium and outflow, which largely exits through the trabecular meshwork and Schlemm canal of the primate eye. Inflow is generally considered to proceed in three steps across the bilayered ciliary epithelium[1-9] (Fig. 1): uptake of solute and water by the pigmented ciliary epithelial (PE) cells at the stromal surface, passage through gap junctions to the nonpigmented ciliary epithelial cell (NPE) layer, and trans-

fer from the NPE cells into the aqueous humor of the anterior chamber. At the stromal surface, paired Na$^+$/H$^+$ and Cl$^-$/HCO$_3^-$ antiports[4,6,10,11] and/or a bumetanide-sensitive Na$^+$-K$^+$-2Cl$^-$ symport[5,8,12-14] can underlie PE-cell uptake of NaCl, the principal solute of the aqueous humor. Which set of mechanisms dominates the first step in secretion has been unclear.

The mouse has been proposed as a potentially useful animal for studying aqueous humor dynamics, because its outflow tract is structurally closer to that of the human[15] than is that of other commonly used nonprimate species, such as the cow or rabbit. We recently adapted an electrophysiologic technique, the servo-null micropipette system (SNMS), for monitoring IOP in the small mouse eye.[16] With the SNMS, we found that IOP responses in the mouse eye parallel those in the human eye, not only to drugs that alter aqueous humor outflow, but also to those that alter aqueous humor inflow.[16] Furthermore, we have found that SNMS measurements of mouse IOP are sufficiently reliable to permit identification of novel receptor mechanisms that regulate IOP.[17] In the current study, we examined IOP responses in the mouse eye to inhibitors of both sets of transport processes implicated in the initial step of formation of aqueous humor.

MATERIALS AND METHODS

Animals

Black Swiss outbred mice of mixed sex, 7 to 9 weeks old and approximately 30 g in weight, were obtained from Taconic, Inc. (Germantown, NY). Animals were housed in accordance with National Institutes of Health recommendations, maintained under a 12-hour light-dark illumination cycle, and allowed unrestricted access to food and water. IOP measurements were performed at the same time of day (2–6 PM) to minimize diurnal effects on IOP. All procedures conformed to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

Anesthesia

Before all IOP measurements, mice received general anesthesia in the form of intraperitoneal ketamine (250 mg/kg), supplemented by topical proparacaine HCl (0.5%; Allergan, Hormigueros, Puerto Rico).[16]

Servo-Null Micropipette System

The SNMS is an electrophysiologic, nonmanometric method of measuring pressure that we have previously adapted and validated for measuring IOP in the mouse.[16] The exploring, 5-μm micropipette is filled with 3 M KCl solution to ensure that the resistance of the fluid within the tip is much lower than that of the extracellular fluid. The resistance to electrical flow through the micropipette is continuously monitored and is dominated by the electrical resistance at the tip. After entry of the tip into the anterior chamber, the step change in hydrostatic pressure forces aqueous humor into the micropipette, displacing the low-resistance 3-M KCl filling solution from the tip back toward the shank. The resultant increase in electrical resistance generates a signal to a vacuum-pressure pump that produces an equal counterpressure that maintains the position of the aqueous humor-KCl interface at the tip of the micropipette and thus sustains the original electrical resistance. This counterpressure equals the hydrostatic pressure outside
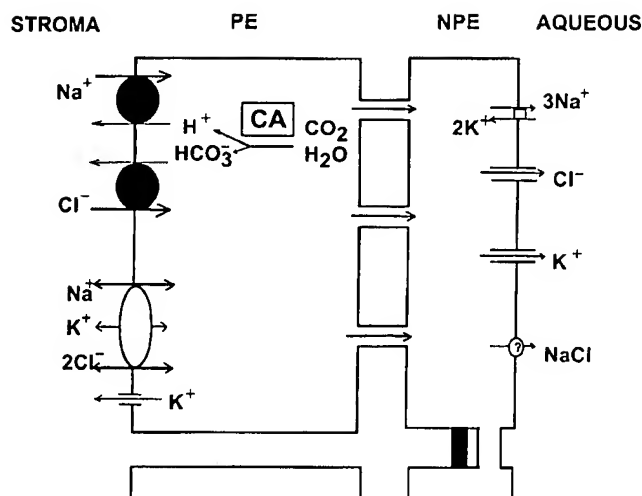
Figure 1. Consensus model of aqueous humor formation (modified from Counillon et al.[22] and McLaughlin et al.[26]). Carbonic anhydrase-limited delivery of H^+ and HCO_3^- limits uptake of stromal NaCl through paired antiports. In parallel, NaCl can also enter (or exit[26]) PE cells through the Na^+-K^+-$2Cl^-$ symport. At the contralateral surface, Na^+ and Cl^- can be released from the NPE cells into the aqueous humor through Na^+,K^+-activated ATPase and Cl^- channels, respectively. An electroneutral transporter may also support release into the aqueous humor.

the micropipette tip, in this instance the IOP. The output signal of the servo-null device (Servo-Null Micropressure System model 900A; World Precision Instruments [WPI], Sarasota, FL) was converted to digital form (Duo 18-Data Recording System; WPI), continuously displayed on a monitor, and saved in a computer file at three to five readings per second. Before every measurement, the system was calibrated externally against a mercury manometer in the range from 0 to 50 mm Hg at 5- to 10-mm Hg intervals.

Micropipette Design

Micropipettes were fabricated from borosilicate glass (1.5 mm outer diameter, 0.84 mm inner diameter, WPI) with a puller (Sutter Instruments, San Rafael, CA). The tips were beveled to an outer diameter of 5 μm and a 45° angle with a micropipette beveler (Sutter). When filled with 3 M KCl solution, these micropipettes displayed resistances of 0.25-0.60 MΩ.

Procedure for Measuring IOP

After reaching a stable plane of anesthesia confirmed by absent response to foot pinch, the mice were secured in a surgical stereotaxic device (David Kopf Instruments, Tujunga, CA), with the head positioned to avoid any pressure on the animal that could affect IOP. A heating pad at 37°C (Delta Phase Isothermal Pad, Braintree Scientific, Braintree, MA) maintained body temperature. Topical proparacaine supplemented general anesthesia, and corneal dehydration was prevented by topical normal saline (309 mOsm), as necessary. The ground electrode was placed on the conjunctiva of the same or the contralateral eye, carefully avoiding any pressure on the eye.

The micropipette tip was next placed in the drop of proparacaine on the cornea overlying the pupil, and the output reading from the SNMS was adjusted to zero. The micropipette was then advanced across the cornea (at 20-30° to the optical axis) into the anterior chamber by a cell-penetration positioning system (model LSS 21200; Burleigh Instruments, Inc., Fishers, NY) and a piezoelectric step driver (model PZ100; Burleigh). IOP was monitored after positioning the micropipette tip in the aqueous humor.

The baseline IOP in the present study was 14.2 ± 0.4 mm Hg (n = 113). In measuring drug-induced changes in IOP, each animal served as

its own series control. All pressures after drug application were compared with those just before the drug was added.

Statistics

To determine an individual IOP reading, the mean ± SEM was calculated during a 3- to 5-minute recording period. Numbers of experiments or eyes are indicated by the symbol n. The statistical significance of changes in IOP was tested with Student's paired *t*-test.

Drugs

Drugs were applied topically in 10-μL droplets with a pipette (Eppendorf; Brinkman Instruments, Westbury, NY) at the stated concentrations; total doses are also provided in parentheses. Agents were initially dissolved in dimethyl sulfoxide (DMSO). Unless otherwise stated, the final droplet solution was an isosmotic saline solution (310 mOsm) containing 1% to 8% DMSO and 0.003% benzalkonium chloride (Sigma Chemical Co., St. Louis, MO), commonly used to enhance ocular drug penetration. We have found that the DMSO-benzalkonium solution itself has no effect on mouse IOP at DMSO concentrations as high as 10% (Table 1). DMSO concentrations as high as 15%[18] to 20%[19] have been reported not to alter IOP in rabbits.

We have already reported evidence that changes in mouse IOP produced by our method of topical administration are mediated by local ocular, and not systemic, actions, because unilateral topical application does not alter either pupillary size (1% pilocarpine,[16] 1% tropicamide[17]) or IOP (100 μM adenosine[17]) in the contralateral eyes. Consistent with our earlier observations, we now report that topical application of 1 mM dimethylamiloride (DMA) did not affect the IOP of the contralateral eye (ΔIOP = 0.08 ± 0.40 mm Hg, n = 6, P > 0.8), but reduced IOP of the treated eye by 3.8 ± 0.5 mm Hg (n = 23, P < 0.001, Table 1).

Among the drugs administered were the selective Na^+/H^+ antiport inhibitors DMA and EIPA (Sigma Chemical Co.). A third such inhibitor used was BIIB723 (Boehringer/Ingelheim, Biberach an der Riss, Germany), which is a member of the BIIB family of Na^+/H^+ antiport blockers.[20] Similar to nearly all other NHE-1 inhibitors, BIIB723 is an acylguanidine, displaying a selectivity for NHE-1 over NHE-2 of approximately 40-fold and an IC_{50} of approximately 30 nM in cardiomyocytes and approximately 100 nM in hamster fibroblasts (Seidler R, unpublished data, 1998-1999). The parent compound (amiloride; Merck, Rahway, NJ) of the amiloride analogues DMA and EIPA is a low-potency inhibitor of both Na^+/H^+ and Na^+/Ca^{2+} antiports and a higher-potency blocker of ENaC Na^+ channels.[21] Bumetanide (Hoffmann-La Roche, Nutley, NJ) is a selective inhibitor of Na^+-K^+-$2Cl^-$ cotransport. Dorzolamide (Trusopt; Merck) is a topical carbonic anhydrase inhibitor.

RESULTS

Single Drug Effects on Mouse IOP

The NHE-1 member of the family of six Na^+/H^+ exchanger (NHE) transporters is known to be the major basis for antiport activity at the basolateral surface of the PE cells facing the stromal surface.[11] DMA, an amiloride analogue with a highly selective inhibitory effect on the NHE-1 antiport,[22] produced a concentration-dependent lowering of IOP (Fig. 2, Table 1). The precise values are uncertain for the threshold droplet concentrations of the drugs used, but DMA was clearly effective at a droplet concentration of 1 mM (2.94 μg, n = 23, Table 1), and a greater lowering of IOP (by 5.0 ± 0.7 mm Hg) was obtained with a droplet concentration of 3 mM (8.82 μg, n = 4; Table 1). Another amiloride analogue, EIPA, displayed the same minimally effective droplet concentration and enhanced lowering of IOP at 3 mM (300 ng; by 4.1 ± 1.0 mm Hg, Table 1). A third acylguanidine antiport inhibitor, BIIB723, produced a maximal hypotensive effect at 3 mM (16.0 μg) of 4.9 ± 1.7 mm Hg,

TABLE 1. Single-Drug Effects of DMA, EIPA, Bumetanide, BIIB723, and Dorzolamide on IOP

Drug	Class	n	Conc.	Dose	ΔIOP (mm Hg)	P
DMA	Na/H antiport inhibitor	3	100 μM	294 ng	+0.9 ± 0.9	
		23	1 mM	2.94 μg	−3.8 ± 0.5	<0.001
		4	3 mM	8.82 μg	−5.0 ± 0.7	<0.01
EIPA	Na/H antiport inhibitor	3	100 μM	300 ng	+0.8 ± 0.2	
		10	1 mM	3.00 μg	−2.6 ± 0.5	<0.001
		6	3 mM	9.00 μg	−4.1 ± 1.0	<0.01
BIIB	Na/H antiport inhibitor	3	10 μM	53.4 ng	−0.4 ± 1.9	
		4	100 μM	534 ng	−2.7 ± 0.4	<0.01
		17	1 mM	5.34 μg	−4.5 ± 0.5	<0.001
		4	3 mM	16.0 μg	−4.9 ± 1.7	
Dorzolamide	CA topical inhibitor	11	55.4 mM	200 μg	−2.9 ± 0.6	<0.001
Bumetanide	Na-K-2Cl symporter blocker	4	10 μM	36.4 ng	−0.2 ± 1.6	
		3	100 μM	364 ng	−0.8 ± 0.7	
		7	1 mM	3.64 μg	−0.7 ± 1.6	
		12	10 mM	36.4 μg	−1.2 ± 0.6	
Contralateral Drugs						
DMA		6	1 mM	2.94 μg	+0.1 ± 0.4	
Vehicle						
DMSO (10%)		5	10%	10.0 μg	−0.3 ± 0.6	

Conc., concentration.

similar to that of DMA ($n = 4$, Table 1), but displayed a lower minimally effective droplet concentration (100 μM [554 ng]), $n = 4$, Table 1). The similarity of the effects of BIIB723 at 1 mM (5.34 μg; −4.5 ± 0.5 mm Hg) and 3 mM (16.0 μg; −4.9 ± 1.7 mm Hg) and the similar reductions produced by all three NHE-1 inhibitors at 3 mM suggest that a maximal IOP reduction was achieved of 4.1 to 5.0 mm Hg. We were unable to increase the delivered droplet concentration without substantially increasing the DMSO level, thereby triggering a vehicle-induced change in IOP.

Carbonic anhydrase inhibition reduces the rate of production of H^+ and HCO_3^-, which in turn must slow the rate of delivery of H^+ and HCO_3^- to all cell sites, including the antiports. We have already reported that inhibiting carbonic anhydrase with intraperitoneal acetazolamide lowers mouse IOP (by 11.9 ± 1.3 mm Hg).[16] We have now found that topical application of dorzolamide also reduces IOP, albeit to a lesser extent at the droplet concentration applied (Table 1).

We also tested the effects of amiloride which inhibits NHE-1 antiports at a potency 1 to 2 orders of magnitude lower than the amiloride analogues DMA and EIPA.[11] Consistent with this information, amiloride itself exerted no significant effect on mouse IOP at a droplet concentration of 1 mM (2.30 μg, $n = 7$, data not shown). To reach a 10-mM concentration, it was necessary to solubilize amiloride in 30% DMSO. After pretreatment with vehicle containing 30% DMSO, subsequent application of 10 mM amiloride in the same concentration of vehicle did not alter that IOP (ΔIOP = −1.0 ± 0.7 mm Hg, $n = 4$, $P > 0.2$). Thus, at a concentration 10 times higher than EIPA's minimal effective concentration, amiloride had no effect, consistent with the known ratio of the potency of these inhibitors (3.9:0.07 μM, or ~56) when applied to PE cells.[11]

In contrast to the IOP reductions triggered by the three selective inhibitors of the NHE-1 antiport at droplet concentrations of 0.1 to 3 mM (Table 1), blockage of the Na^+-K^+-$2Cl^-$ symport with droplet concentrations of 0.1 to 10 mM (364 ng to 36.4 μg) bumetanide had no significant effect on IOP (Fig. 2, Table 1).

Sequential Drug Effects on Mouse IOP

Electron microprobe analyses[6] have suggested that inhibition of the Na^+-K^+-$2Cl^-$ symport lowers Cl^- uptake by the ciliary epithelium under conditions in which the turnover rate of the
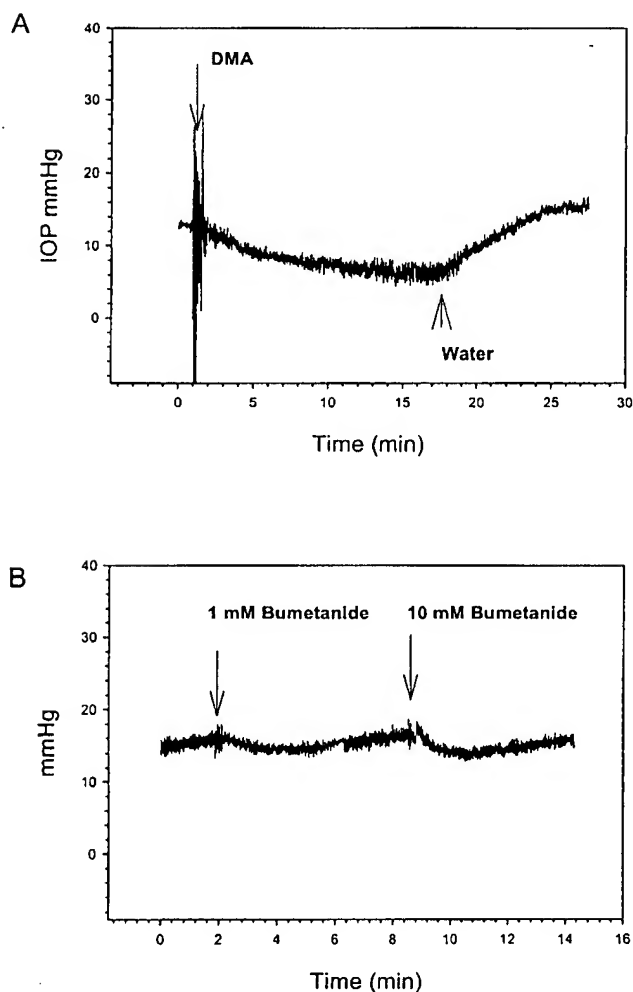

FIGURE 2. Responses of mouse IOP to inhibition of Na^+/H^+ antiports with DMA or to inhibition of Na^+-K^+-$2Cl^-$ antiports with bumetanide. (A) DMA (1 mM, 2.94 μg) lowered IOP. Water was added at the conclusion of this and many other experiments to verify the patency of the micropipette by osmotically raising IOP.[16] (B) Neither 1 mM (3.64 μg) nor 10 mM (36.4 μg) bumetanide by itself significantly altered mouse IOP.
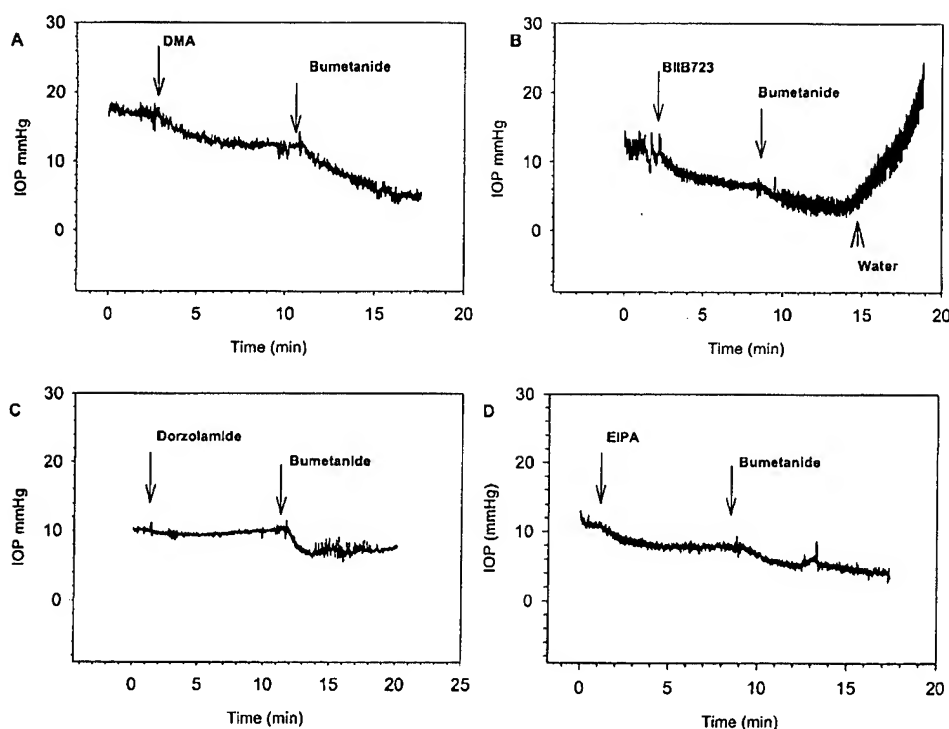
FIGURE 3. Responses to sequential topical addition of direct or indirect inhibitors of Na^+/H^+ antiports, followed by bumetanide: (A) 1 mM (2.94 μg) DMA followed by 1 mM (3.64 μg) bumetanide, (B) 1 mM (5.34 μg) BIIB723 followed by 1 mM (3.64 μg) bumetanide, (C) 55.4 mM (200 μg) dorzolamide followed by 1 mM (3.64 μg) bumetanide, and (D) 1 mM EIPA (3.00 μg) followed by 1 mM (3.64 μg) bumetanide. In each case, bumetanide significantly reduced IOP after prior inhibition of the Na^+/H^+ antiport.

Na^+/H^+ antiport is reduced. To test this hypothesis in vivo, we applied bumetanide after first reducing Na^+/H^+ antiport exchange either directly with acylguanidine inhibitors or indirectly with a carbonic anhydrase inhibitor (Fig. 3, Table 2).

In each case, topical application of the first drug produced the anticipated significant decrease in IOP. Thereafter, the same 10-mM droplet concentration (36.4 μg) of bumetanide, which was ineffective by itself, now triggered significant further lowering of IOP. The entries in Table 2 present the changes in IOP produced first by the initial drug (with respect to baseline) and second by the later addition of bumetanide (in comparison with the previous experimental period). In every case, the secondary application of bumetanide reduced IOP by 3.8 to 4.0 mm Hg (Table 2). Directly inhibiting the Na^+/H^+ antiport with a submaximal 1-mM concentration (5.34 μg) of BIIB723 slightly enhanced the reduction in IOP previously triggered by indirectly inhibiting the antiport with dorzolamide (ΔIOP = -0.7 ± 0.2 mm Hg, Table 2).

DISCUSSION

The salient findings of the present study are that separate topical application of three different acylguanidine inhibitors

of the NHE-1 Na^+/H^+ antiport reduced IOP at 1-mM droplet concentrations, but the far less potent parent compound (amiloride) had no effect on IOP at tenfold higher concentration; application of the selective Na^+-K^+-$2Cl^-$ symport inhibitor bumetanide itself had no significant effect; topical application of the carbonic anhydrase inhibitor dorzolamide reduced IOP in the mouse; and after first inhibiting the NHE antiports either directly with acylguanidine blockers or indirectly with dorzolamide, the subsequent application of bumetanide triggered a highly significant further reduction in IOP of 3.8 to 4.0 mm Hg.

As discussed elsewhere,[17] we do not know the drug concentrations in the very small volume of the mouse anterior chamber (2-4 μL[16,23]) after topical application. However, comparisons of minimally effective droplet concentrations of purinergic drugs with their published K_i suggest that the penetrance (defined as the aqueous-to-droplet concentration ratio) is commonly approximately 1:100 to 1:1000.[17] To extrapolate these values for purinergic drugs to the acylguanidine blockers and bumetanide is necessarily speculative. However, as discussed elsewhere,[17] this apparent penetrance of drugs in the mouse eye is not very different from the approximately 1:100

TABLE 2. Effects on IOP of Sequential Medications

First Drug Second Drug	n	Conc. of First Drug/ Second	ΔIOP (mm Hg) (after baseline)	P	ΔIOP (mm Hg) (after first drug)	P
Dorzolamide (CA inhibitor)/	4	55.5 mM (200 μg)/	-2.0 ± 0.4	<0.05		
Bumetanide (symport inhibitor)		10 mM (36.4 μg)			-3.9 ± 1.0	<0.05
BIIB (Na^+/H^+ antiport inhibitor)/	6	1 mM (5.34 μg)/	-2.9 ± 1.0	<0.05		
Bumetanide (symport inhibitor)		10 mM (36.4 μg)			-3.9 ± 0.9	<0.01
DMA (Na^+/H^+ antiport inhibitor)/	6	1 mM (2.94 μg)/	-4.0 ± 0.8	<0.01		
Bumetanide (symport inhibitor)		10 mM (36.4 μg)			-3.8 ± 0.7	<0.01
EIPA (Na^+/H^+ antiport inhibitor)/	6	1 mM (3.00 μg)/	-2.4 ± 0.6	<0.01		
Bumetanide (symport inhibitor)		10 mM (36.4 μg)			-4.0 ± 0.6	<0.01
Dorzolamide (CA inhibitor)/	7	55.4 mM (200 μg)/	-3.5 ± 0.9	<0.01		
BIIB (Na/H antiport inhibitor)		1 mM (5.34 μg)			-0.7 ± 0.2	<0.01

Conc., concentration.

penetrance of drugs topically applied to rabbits and primates, as well. By this measure, the minimally effective droplet concentration of 1 mM for DMA and EIPA (Table 1) may have corresponded to approximately 1 to 10 μM in the aqueous humor, and the minimally effective droplet concentration of 100 μM for BIIB723 may have corresponded to aqueous humor concentrations of 0.1 to 1 μM. This difference may arise from a higher penetrance for BIIB723, because the IC_{50} observed for this drug (30-100 nM; Seidler R, unpublished results, 1998-1999) is similar to that of EIPA (50 nM[24]). Although BIIB723 may penetrate more effectively than DMA or EIPA, it is likely that all three NHE-1 inhibitors exerted a maximal effect at 3 mM (see first paragraph of Results), uniformly reducing IOP by 4.1 to 5.0 mm Hg.

The first step in aqueous humor formation is electroneutral uptake of NaCl from the stroma of the ciliary processes by the PE cells of the ciliary epithelium and can be mediated by either paired NHE-1 Na^+/H^+ and AE2 Cl^-/HCO_3^- exchangers[4,6,10,11] or an Na^+-K^+-$2Cl^-$ cotransporter.[3,8,12-14] Consensus has not yet been reached concerning the relative importance of these two transfer mechanisms. However, electron probe x-ray microanalyses of the elemental compositions of rabbit ciliary epithelium in vitro have suggested that the paired antiports can predominate, at least under certain conditions, and that the bumetanide-sensitive symport can support either uptake or release of solute, depending on the ambient thermodynamic driving force.[6] This interpretation is consistent with the observation that inhibition of the Na^+-K^+-$2Cl^-$ symport with bumetanide has no significant effect on inflow or IOP in the cynomolgus monkey.[25] However, the putative role of Na^+/H^+ and Cl^-/HCO_3^- antiports in regulating mammalian IOP has not previously been tested in vivo.

In the present work, we tested three predictions based on the microprobe analyses.[6] First, if the paired antiports are the dominant mechanism in the first step of aqueous humor formation, blocking one or the other antiport should reduce inflow and thereby IOP. This prediction was met by the oculohypotensive effects of three different acylguanidine NHE-1 inhibitors (Fig. 2, Table 1). Second, if the Na^+-K^+-$2Cl^-$ symport plays a supplemental role in supporting either uptake or release at the stromal surface, blocking the symport would be expected to have little effect on inflow. Consistent with this prediction, we have confirmed in the mouse that bumetanide alone has no significant effect on IOP, in agreement with the earlier observation in cynomolgus monkeys.[25] Third, when the paired activity of the antiports is blocked, the major mechanism supporting NaCl uptake from the stroma should be the Na^+-K^+-$2Cl^-$ symport. Under these conditions, bumetanide is predicted to have a substantial effect on secretion (see Figures 2 and 3 of McLaughlin et al.[6]). Indeed, the same concentration of bumetanide which was by itself ineffective now uniformly reduced mouse IOP, after either direct NHE inhibition with the acylguanidine compounds or after the carbonic anhydrase inhibitor dorzolamide, which probably inhibits NHEs indirectly by reducing delivery of H^+ and HCO_3^- to the antiports. The IOP recordings in the current study, limited to 12 to 20 minutes largely because of the general anesthesia requirement, establish roles for the antiports, but additional research is needed to learn whether antiport inhibition is an effective strategy for long-term IOP control.

IOP reflects both the inflow and outflow of aqueous humor. Because present methodology permits only IOP measurements in the mouse, the current results can neither exclude an outflow effect nor unambiguously prove that the paired NHE-1 Na^+/H^+ and AE2 Cl^-/HCO_3^- antiports are the dominant mechanisms underlying the first step in formation of aqueous humor. However, the data are consistent with the latter antiport hypothesis and further lead to the proposal that bumet-

anide can have a previously unobserved role in lowering IOP if coupled to inhibition of the NHE exchangers.

References

1. Chu TC, Candia OA. Electrically silent Na+ and Cl− fluxes across the rabbit ciliary epithelium. *Invest Ophthalmol Vis Sci.* 1987;28: 445-450.

2. Carre DA, Tang CS, Krupin T, Civan MM. Effect of bicarbonate on intracellular potential of rabbit ciliary epithelium. *Curr Eye Res.* 1992;11:609-624.

3. Edelman JL, Sachs G, Adorante JS. Ion transport asymmetry and functional coupling in bovine pigmented and nonpigmented ciliary epithelial cells. *Am J Physiol.* 1994;266:C1210-C1221.

4. Kaufman PL, Mittag TW. *Textbook of Ophthalmology.* Vol 7. *Glaucoma.* St. Louis: Mosby-Year Book; 1994.

5. Wolosin JM, Candia OA, Peterson-Yantorno K, Civan MM, Shi XP. Effect of heptanol on the short circuit currents of cornea and ciliary body demonstrates rate limiting role of heterocellular gap junctions in active ciliary body transport. *Exp Eye Res.* 1997;64: 945-952.

6. McLaughlin CW, Peart D, Purves RD, et al. Effects of $HCO3^-$ on cell composition of rabbit ciliary epithelium: a new model for aqueous humor secretion. *Invest Ophthalmol Vis Sci.* 1998;39: 1631-1641.

7. Walker VE, Stelling JW, Miley HE, Jacob TJ. Effect of coupling on volume-regulatory response of ciliary epithelial cells suggests mechanism for secretion. *Am J Physiol.* 1999;276:C1432-C1438.

8. Crook RB, Takahashi K, Mead A, Dunn JJ, Sears ML. The role of NaKCl cotransport in blood-to-aqueous chloride fluxes across rabbit ciliary epithelium. *Invest Ophthalmol Vis Sci.* 2000;41:2574-2583.

9. To CH, Do CW, Zamudio AC, Candia OA. Model of ionic transport for bovine ciliary epithelium: effects of acetazolamide and HCO. *Am J Physiol.* 2001;280:C1521-C1530.

10. Wiederholt M, Helbig H, Korbmacher C. Ion transport across the ciliary epithelium: lessons from cultured cells and proposed role of carbonic anhydrase. In: Botré F, Gros G, Storey BT, eds. *Carbonic anhydrase: from biochemistry and genetics to physiology and clinical medicine. Proceedings of the International Workshop on Carbonic Anhydrase, Spoleto, Italy, March 1990.* New York: VCH; 1991;xvi,467.

11. Counillon L, Touret N, Bidet M, et al. Na+/H+ and Cl−/HCO3− antiporters of bovine pigmented ciliary epithelial cells. *Pflügers Arch.* 2000;440:667-678.

12. Wiederholt M, Zadunaisky JA. Membrane potentials and intracellular chloride activity in the ciliary body of the shark. *Pflügers Arch.* 1986;407:S112-S115.

13. Do CW, To CH. Chloride secretion by bovine ciliary epithelium: a model of aqueous humor formation. *Invest Ophthalmol Vis Sci.* 2000;41:1853-1860.

14. Dunn JJ, Lytle C, Crook RB. Immunolocalization of the Na-K-Cl cotransporter in bovine ciliary epithelium. *Invest Ophthalmol Vis Sci.* 2001;42:343-353.

15. Tamm ER, Russell P, Piatigorsky J. Development of characterization of a immortal and differentiated murine trabecular meshwork cell line. *Invest Ophthalmol Vis Sci.* 1999;40:1392-1403.

16. Avila MY, Carre DA, Stone RA, Civan MM. Reliable measurement of mouse intraocular pressure by a servo-null micropipette system. *Invest Ophthalmol Vis Sci.* 2001;42:1841-1846.

17. Avila MY, Stone RA, Civan MM. A(1)-, A(2A)- and A(3)-subtype adenosine receptors modulate intraocular pressure in the mouse. *Br J Pharmacol.* 2001;134:241-245.

18. Crosson CE. Adenosine receptor activation modulates intraocular pressure in rabbits. *J Pharmacol Exp Ther.* 1995;273:320-326.

19. Crosson CE. Intraocular pressure responses to the adenosine agonist cyclohexyladenosine: evidence for a dual mechanism of action. *Invest Ophthalmol Vis Sci.* 2001;42:1837-1840.

20. Wu D, Stassen JM, Seidler R, Doods H. Effects of BIIB513 on ischemia-induced arrhythmias and myocardial infarction in anesthetized rats. *Basic Res Cardiol.* 2000;95:449–456.

21. Kleyman TR, Cragoe EJ Jr. Amiloride and its analogs as tools in the study of ion transport. *J Membr Biol.* 1988;105:1–21.

22. Counillon L, Scholz W, Lang HJ, Pouyssegur J. Pharmacological characterization of stably transfected Na+/H+ antiporter isoforms using amiloride analogs and a new inhibitor exhibiting anti-ischemic properties. *Mol Pharmacol.* 1993;44:1041–1045.

23. Lea GS, Amann S, Chakravarti J, Lass J, Edelhauser HF. Intraocular volumes and surface area measurements of the mouse eye: wild type vs lumican-deficient [ARVO Abstract]. *Invest Ophthalmol Vis Sci.* 2000;41(4):S739. Abstract nr 3934.

24. Scholz W, Albus U, Counillon L, et al. Protective effects of HOE642, a selective sodium-hydrogen exchange subtype 1 inhibitor, on cardiac ischaemia and reperfusion. *Cardiovasc Res.* 1995; 29:260–268.

25. Gabelt BT, Wiederholt M, Clark AF, Kaufman PL. Anterior segment physiology after bumetanide inhibition of Na-K-Cl cotransport. *Invest Ophthalmol Vis Sci.* 1997;38:1700–1707.

26. McLaughlin CW, Zellhuber-McMillan S, Peart D, Purves RD, Macknight ADC, Civan MM. Regional differences in ciliary epithelial cell transport properties. *J Membr Biol.* 2001;182:213–222.

(dT)$_{n-0}$ and poly(rC) · oligo(dG)$_{12-18}$. Ten microliters of virus (which was concentrated 100-fold by centrifugation and then suspended in 50 mM tris-HCl, pH 7.3, 10 mM dithiothreitol, and bovine serum albumin, 1 mg/ml) were incubated with 10 µl of 50 mM tris-HCl, pH 7.3, 10 mM dithiothreitol, 0.06 percent Triton X-100 for 10 minutes at 2°C. A portion (20 µl) of assay mixture was added, and the resulting 40-µl reaction mixture was incubated for 60 minutes at 37°C. The reaction was stopped by the addition of 0.5 ml of 80 mM sodium pyrophosphate, 25 µl of bovine serum albumin (5 mg/ml), and 0.5 ml of 25 percent trichloroacetic acid at 2°C. Acid-insoluble material was collected on glass fiber filters, washed extensively with 10 percent trichloroacetic acid, dried, and dissolved in NCS tissue solubilizer; the radioactivity was then counted in toluene-based scintillation fluid. The reaction mixtures with poly(rA) · oligo(dT)$_{12-18}$ and poly(dA) · oligo (dT)$_{12-18}$ consisted of: 50 mM tris-HCl, pH 7.3, 70 mM KCl, 10 mM dithiothreitol, 0.1 mM each of deoxyriboadenosine triphosphate (dATP), deoxyriboguanosine triphosphate (dGTP), deoxyribocytidine triphosphate (dCTP), 2.3 µM [³H]-labeled thymidine triphosphate (40,000 count/min per picomole), 20 µg of poly(rA) or poly(dA) per milliliter, 20 µg of oligo(dT)$_{12-18}$ per milliliter, and either 5 mM MgCl₂ or 0.4 mM MnCl₂. The reaction mixture containing poly(rC) · oligo(dG)$_{12-18}$ consisted of 50 mM tris-HCl, pH 7.3, 50 µg of actinomycin D per milliliter, 15 mM KCl, 10 mM dithiothreitol, 10.7 µM [³H]-labeled dGTP (6500 count/min per picomole), 40 µg of poly(rC) per milliliter, 40 µg of oligo(dG)$_{12-18}$ per milliliter, and either 25 mM MgCl₂ or 0.4 mM MnCl₂.

11. R. C. Nowinski, E. Edynak, N. H. Sarkar, Proc. Natl. Acad. Sci. U.S.A. 68, 1608 (1971); H. C. Chopra, I. Zelljadt, E. M. Jensen, M. M. Mason, N. J. Woodside. J. Natl. Cancer Inst. 46, 127 (1971).
12. N. C. Goodman and S. Spiegelman, Proc. Natl. Acad. Sci. U.S.A. 68, 2203 (1971).
13. J. W. Abrell and R. C. Gallo, J. Virol. 12, 431 (1973).
14. J. Schlom and S. Spiegelman, Science 174, 840 (1971).
15. Serum was neutralized by titrating the virus on FC/2Th cells before and after reacting serial tenfold dilutions of virus with an equal volume of antiserum diluted 1 : 5 at room temperature for 1 hour. Inoculated cultures were split 1 : 10 by trypsinization at weekly intervals. After two passages, RDDP activity in the culture fluids was determined. The titer of the virus was 10⁶·³ tissue culture infective doses (50 percent effective) per 0.1 ml with and without serum. The antiserum used was prepared in goats against intact virus and was obtained from Dr. R. Wilsnack, Huntingdon Research Center, Brooklandville, Md. Mouse mammary tumor virus, baboon type C virus (M7), and woolly monkey sarcoma virus antiserums from the same source also failed to neutralize the squirrel monkey virus when similarly tested.
16. P. K. Nakane and G. B. Pierce, J. Histochem. Cytochem. 14, 929 (1966); E. H. Leduc, S. Wicker, S. Avrameas, W. Bernhard, J. Gen. Virol. 4, 609 (1969).
17. The rabbit antiserum to M-PMV polymerase was supplied by Dr. M. Ahmed, Pfizer Inc., Maywood, N.J.
18. K. H. Rand and C. Long, Nature New Biol. 240, 187 (1972).
19. M. Ahmed, G. Schidlovsky, D. L. Larson, M. Mancuso, S. A. Mayyasi, J. Gen. Virol. 30, 11 (1976).
20. A. F. Bykovsky, I. S. Irlin, V. M. Zhdanov, Arch. Gesamte Virusforsch. 45, 144 (1974).
21. R. S. Howk, L. A. Rye, L. A. Killeen, E. M. Scolnick, W. P. Parks, Proc. Natl. Acad. Sci. U.S.A. 70, 2117 (1973); D. H. Moore, Cancer Res. 34, 2322 (1974); A. S. Dion, A. B. Vaidya, G. S. Fout, ibid., p. 3509; D. L. Fine and L. O. Arthur, Abstr. Annu. Mtg. Am. Soc. Microbiol. (1974), p. 246 (V273); R. Michalides, J. Schlom, J. Dahlberg, K. Park, J. Virol. 16, 1039 (1975).
22. A. Hellman et al., J. Virol. 14, 133 (1974); K. H. Rand, C. W. Long, T. T. Wei, R. V. Gilden, J. Natl. Cancer Inst. 53, 449 (1974).
23. J. Schlom, G. Schochetman, D. Colcher, W. Drohan, Abstracts, VII International Symposium on Comparative Leukemia Research No. C-11 (1975); D. Colcher, W. Drohan, J. Schlom, J. Virol. 17, 705 (1976).
24. Supported by contract NO1-CP-43214 within the Virus Cancer Program, National Cancer Institute. We thank A. Bodenman, C. Thomas, and G. Peacher for technical assistance.

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Sodium-Specific Membrane Channels of Frog Skin Are Pores: Current Fluctuations Reveal High Turnover

Abstract. The reversible sodium transport blocker amiloride causes current fluctuations at the apical membrane of the outer stratum granulosum of frog skin. Their power density spectra reveal that single transport sites translocate more than 10^6 sodium ions per second, which indicates a pore mechanism. The density of open plus amiloride-blocked pores is in the order of 10^8 pores per square centimeter of skin area with 60 millimolar sodium and 18 micromolar amiloride in the outer solution.

Carrier- and pore-mediated transport through biological membranes can be distinguished by the turnover number of individual transport sites (1). While a carrier molecule, which has to move through the lipid phase of the membrane, is not likely to transport more than 10^4 ions or molecules per second, the transport rate of a pore can be several orders of magnitude larger (2). Applying this idea to the Na-selective membrane of frog skin, we have attempted to determine the Na turnover of individual transport sites by an evaluation of current fluctuations. The fluctuations were introduced artificially by addition of the drug amiloride, a pyrazine diuretic known to block Na transport reversibly from the outside (3). A reversible blocker can be expected to randomly interrupt the Na turnover of individual transport sites. A site will then either conduct fully or, when blocked, not conduct at all. The continuous current i passing one site is thus chopped up into small current pulses of varying duration but equal amplitude (i). The pulses add up to a mean current which on close inspection will show random fluctuations. The mean current per square centimeter will be

$$I_{Na} = iMP_o \qquad (1)$$

where M (cm^{-2}) is the mean density of unblocked plus amiloride-blocked transport sites. The steady-state probability P_o represents the fraction of M not blocked by amiloride and MP_o the mean density of open sites. Statistical evaluation of the fluctuations permits computation of i and thus the Na turnover of individual transport sites in the open state.

Isolated abdominal skin of *Rana esculenta* was used at room temperature. It was mounted in a Lucite chamber which left 3 cm² exposed to the bathing solutions. The outer solution was K or Na sulfate Ringer solution containing varying concentrations of amiloride. The inner one was K sulfate Ringer solution, which can be expected to depolarize the K-selective inward-facing membranes of the epithelium and to increase their conductance. Thus, transepithelial resistance and potential were largely determined by the apical membrane of the stratum granulosum (4). The current component that did not pass the Na-specific channels was determined as the current flowing in the presence of 35 µM amiloride, and was subtracted from the total current to obtain the transcellular Na current, I_{Na}.

Transepithelial voltage was clamped to 0 mv by a voltage clamp circuit with continuous feedback. The input stage of the voltage-sensing amplifier was designed around a matched pair of low-noise transistors (National Semiconductor 2N4250) to minimize feedback current fluctuations arising from this stage. The open-loop clamp gain was 25,000. The short-circuit current was amplified with a gain of 50 µv/na, fed through a high-pass RC-filter with a characteristic frequency of 0.007 hertz, amplified 400 times, and recorded on magnetic tape. Recording periods were 10 to 30 minutes for each amiloride concentration.
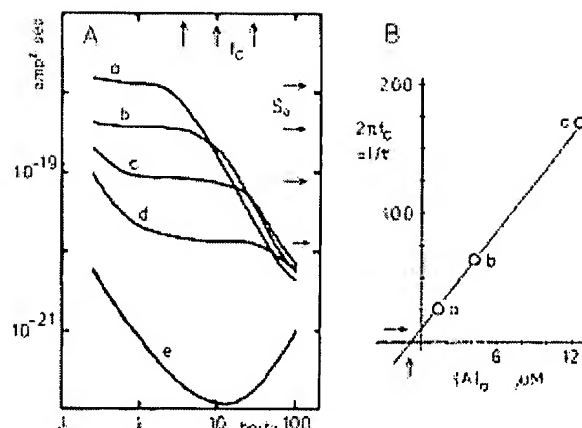
The amplified a-c current signal was sampled from the magnetic tape at frequencies of 50 and 5000 hertz by use of an anti-aliasing filter of the Butterworth type (72 db per octave, characteristic frequency set at 80 percent of the maximal analyzed frequency). The digitized signal was divided into 20 records of 4096 words each. A power density spectrum was computed from each record on an IBM 370/58 by use of a fast Fourier transform program. The spectra of 20 records were averaged.

Figure 1A shows five power density spectra in the range 0.3 to 100 hertz. Curves a to d were obtained with a sodium activity of $(Na)_o = 60$ mM in the outer solution and amiloride concentrations of $(A)_o = 1.4, 4.3, 12.7,$ and 35 µM. Spectrum e was obtained with $(Na)_o = 0$. Spectra a to d are of the Lorentz type expected for exponential relaxation phenomena. They obey the relationship
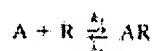
$$S = S_o/[1 + (f/f_c)^n] \qquad (2)$$

where S is the power density (amp² sec), S_o the plateau value, f the frequency (hertz), and f_c the corner frequency. The exponent n was found to be in the range 1.75 to 2 (1.8 in Fig. 1A). It is evident

Fig. 1. (A) Power density spectra obtained from 3 cm² of epithelium. Only the parts of the recorded spectra that are relevant for the arguments of this report are shown. (Curves a to d) Increasing amiloride concentrations of $(A)_o = 1.4, 4.3, 12.7,$ and 35 μM at 60 mM $(Na)_o$. (Curve e) Sodium-free outer solution (Na replaced by K). The corresponding values of transcellular Na current are (from curve a to curve e) 41, 24, 9, 0, and 0 μa per 3 cm². The transcellular Na current at $(A)_o = 0$ was 78 μa per 3 cm² (spectrum not shown). Plateau values (S_o) and corner frequencies (f_c) are indicated with horizontal and vertical arrows; f_c was read off where the power density equaled half of the plateau value. The f_c of curve d (for which I_{Na} was zero) was not evaluated because the a value of this curve fell clearly below the expected range. (B) Relationship between corner frequency and amiloride concentration (see Eq. 4a). Arrows point to the intercepts where k_2 and K_A' are read off. The linear dependence of $1/\tau$ on $(A)_o$ shows, in retrospect, that neglecting the term $k_1(R)$ in Eq. 4 was justified.

that the plateau value, S_o, of the power spectrum decreases with increasing amiloride concentration while the corner frequency f_c increases.

Following a derivation in Verveen and DeFelice (5), we may set $2\pi f_c$ equal to the chemical rate $(1/\tau)$ of the current-modulating process. In the simplest possible case this process is due to the random formation and disintegration of a transport-blocking complex between amiloride (A) and the transport site (R)

$$A + R \underset{k_1}{\overset{k_1}{\rightleftharpoons}} AR$$

where AR is the blocked site; k_1 and k_2 are rate constants, and their ratio $K_A = k_2/k_1$ is the dissociation constant of AR. With $M = R + AR$, where R and AR are the densities of unblocked and blocked sites, we find from the law of mass action the steady-state probabilities that a site is unblocked or blocked

$$P_o = R/M = 1/[1 + (A)_m/K_A]$$
$$P_A = AR/M = P_o(A)_m/K_A \quad (3)$$

where $(A)_m$ is the effective amiloride concentration at the outer surface of the membrane. This blocking mechanism implies the following relationship between chemical rate and equilibrium concentrations of A and R (6)

$$1/\tau = k_1(A)_m + k_1(R) + k_2 \quad (4)$$

in which (R) is the equilibrium concentration of unblocked transport sites, expressed in the same units as $(A)_m$. In cases where (R), which decreases with increasing $(A)_m$, is numerically negligible compared to $(A)_m$, a linear relationship between amiloride concentration and chemical rate will be expected. We thus obtain the prediction

$$2\pi f_c = 1/\tau = k_1(A)_m + k_2 =$$
$$k_1\beta(A)_o + k_2 \quad (4a)$$

where $\beta = (A)_m/(A)_o$ is the unknown partition coefficient of amiloride between the outer bulk solution and the space at the outer surface of the membrane. After substituting β into Eq. 3 and combining Eq. 3 with Eq. 4a, we find the "on" and "off" probabilities to be

$$P_o = 1/[1 + (A)_o/K_A'] = k_2\tau$$
$$P_A = P_o(A)_o/K_A' = k_1'\tau(A)_o \quad (3a)$$

The linear relationship between f_c and $(A)_o$ predicted by Eq. 4a was found in most experiments (for example, see Fig. 1B). This shows that channel blocking by amiloride can be described in terms of simple, bimolecular kinetics, and that $k_1(R)$ in Eq. 4 is numerically negligible. The rate constant k_2 and the "apparent" constants $k_1' = k_1\beta$ and $K_A' = K_A/\beta$ can be estimated from the plot. We found $1/k_2$, the mean lifetime of the complex AR, to be in the order of 100 msec at room temperature. The apparent dissociation constant K_A' was close to 1 μM at 60 mM $(Na)_o$. It decreased with decreasing $(Na)_o$, as expected if Na and amiloride compete for transport sites (7). The apparent constant k_1' was found to be in the order of 10^7 liter mole^{-1} sec^{-1} at 60 mM $(Na)_o$.

For randomly blocked transport sites with unequal on and off probabilities (5) the plateau value of the power density spectrum (Eq. 2) is, in our notation, given by

$$S_o = 4Mi^2P_oP_A\tau a \quad (5)$$

in which a is the experimental membrane area. Substituting Eq. 1 into Eq. 5, we obtain

$$S_o = 4I_{Na}P_A\tau/a \quad (5a)$$

Further combination with Eqs. 3a and 4a yields an expression for the amplitude of individual current pulses

$$i = \frac{S_o}{4aI_{Na}} \cdot \frac{k_1\beta}{(A)_o}[(A)_o + K_A']^2 \quad (6)$$

For $(Na)_o = 60$ mM, values in the range 0.3 to 0.5 pa were computed, which correspond to turnover numbers of 1 to 3 × 10^6 Na ions per second for individual transport sites. These turnover numbers are much larger than the values of 10^4 ion/sec which are expected (J) and were observed (8) for shuttle-type mobile carriers, like valinomycin, which have to diffuse through the lipid phase of the membrane. Turnover rates of nonshuttle-type carriers, where the carrier molecule rotates or where only a part of the carrier molecule moves, have, to our knowledge, not yet been obtained experimentally. However, since such carriers will also have to overcome the viscosity and the electrostatic energy barrier of the lipid phase, low turnover rates will be expected in this case too. We feel justified, therefore, in concluding that in the Na-selective membrane of frog skin, transport occurs through pores, which are the only high-rate translocators presently known (9). This result is at variance with a previous conclusion by Biber and Sanders (10), who maintained that the Na transport is carrier mediated.

The mean density of open plus amiloride-blocked Na pores can be calculated from

$$M = I_{Na}/(iP_o) = 4aI_{Na}^2P_A\tau/(P_oS_o) \quad (7)$$

It was found to be in the range 0.7 to 2 × 10^8 pores per square centimeter of membrane area at 60 mM $(Na)_o$. When $(Na)_o$ was lowered to 15 mM by substitution with K, larger pore densities were computed, but did not exceed 3 × 10^8 pores per square centimeter. Extrapolation to $(Na)_o \to 0$ shows that the total number of pores (N) will be below 5 × 10^8 cm^{-2}. This density corresponds to less than 50 pores per square micrometer of membrane area if a homogeneous distribution of pores over the apical membranes of all cells of the outer stratum granulosum may be assumed.

If, in the absence of amiloride, all pores were permanently open, a Na current $I_{Na} = iN = 2000\ \mu a/cm^2$ would result at 60 mM (Na)$_o$. This current is 75 times larger than the I_{Na} value of 26 $\mu a/cm^2$ actually observed (see legend to Fig. 1A). We deduce from this observation that even in the absence of amiloride the pores are not permanently open (*11*).

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References and Notes

1. C. M. Armstrong. *Biophys. J.* 15, 932 (1975); *Q. Rev. Biophys.* 7, 179 (1975).
2. We suggest that (i) the term carrier be applied only to translocators where the transfer-mediating moiety moves through the lipid phase; (ii) the term pore be applied to water-filled pathways that permit diffusional transport, which can, however, involve binding of ions to parts of the pore structure; and (iii) different terms be used for translocators that do not meet these specifications [see also (9)].
3. E. N. Ehrlich and J. Crabbé, *Pflügers Arch.* 302, 79 (1968).
4. Under short-circuit conditions, the voltage across the apical membrane will come even closer to the transepithelial voltage when the resistance of the apical membrane is increased by amiloride. Also, the decrease of I_{Na} due to amiloride will decrease the K current of the K-selective, inward-facing membrane, and thus decrease the possible effect of this membrane on the recorded fluctuations. Finally, the intracellular Na concentration can be expected to become small in the presence of amiloride. This condition simplifies the computation of channel conductance.
5. A. A. Verveen and L. J. DeFelice. *Prog. Biophys. Mol. Biol.* 28, 189 (1974).
6. G. H. Czerlinski. *Chemical Relaxation* (Dekker, New York, 1966); G. G. Hammes, *Adv. Protein Chem.* 23, 1 (1968).
7. A. W. Cuthbert and W. K. Shum, *Naunyn Schmiedebergs Arch. Pharmakol.* 281, 261 (1974); *Proc. R. Soc. London Ser. B* 189, 543 (1975).
8. P. Läuger, "Carrier-mediated ion transport," *Science* 178, 24 (1972).
9. Theoretically, translocators that combine properties of carriers and pores are conceivable. For instance, at a transport site the membrane may effectively be thinned down to a narrow protein structure, part of which can bind an ion selectively and transfer it by a rotational movement in low-viscosity surroundings ["transhaese"; see P. Mitchell, *Nature (London)* 180, 134 (1957)]. Transfer rates may be high, although this is not very likely in view of the low turnover numbers of most enzymes. H. Passow (personal communication) has estimated turnover numbers of $2 \times 10^6\ sec^{-1}$ for the anion "carrier" of erythrocyte membranes, which might fulfill the structural requirements mentioned above. It is an open question of nomenclature how such translocators are to be classified. They resemble pores because the larger part of the membrane's diameter is passed by diffusion through a hydrophilic channel, and they resemble carriers because movement of a membrane component is essential for the transfer event. In the spirit of (2) we would not classify such structures, if they exist, as carriers. They may be viewed as pores in which the selectivity filter [B. Hille, *J. Gen. Physiol.* 58, 599 (1971)] constitutes a peculiar energy barrier.
10. T. U. L. Biber and M. L. Sanders, *J. Gen. Physiol.* 61, 529 (1973).
11. A. W. Cuthbert [*J. Physiol. (London)* 228, 681 (1973)] computed, from amiloride binding data, a channel density of 400 μm^{-2} [lower limit at 2.5 mM (Na)$_o$], and from this calculated turnover rates for individual Na channels of 2000 sec^{-1} at 2.5 mM (Na)$_o$ and 8000 sec^{-1} at 115 mM (Na)$_o$ (upper limits). These turnovers are integrated over open and closed times of a channel and therefore cannot be directly compared with our results.
12. This work was supported by Deutsch Forschungsgemeinschaft as project C1 within SFB 38, and by the Humboldt Foundation.

14 June 1976; revised 19 August 1976

Size Limit of Molecules Permeating the Junctional Membrane Channels

Abstract. *The permeability of the cell-to-cell membrane channels in salivary gland cell junction (Chironomus thummi) was probed with fluorescent-labeled amino acids and synthetic or natural peptides. Molecules up to 1200 daltons pass through the channels with velocities depending on molecular size. Molecules of 1900 daltons or greater do not pass. This passage failure seems to reflect the normal size limit for junctional channel permeation; the channels continue to be permeated by the molecules up to 1200 daltons when these are mixed with the nonpermeant molecules. From this size limit a channel diameter of 10 to 14 angstroms is estimated.*

Since the finding that fluorescein, a molecule of 330 daltons, passes through a cell junction of low electrical resistance (*1*), other fluorescent and colorant substances have been used to probe junctional permeability properties (*2, 3*). However, the range of questions that could be studied has been restricted by the small number of useful probes available. We have now enlarged the repertoire of probes with the aim of determining the size limit of molecules permeating the junctional membrane channels (*4*).

We set out to construct fluorescent conjugates which incorporate some of the desirable features of the popular tracer fluorescein, such as water solubility, nontoxicity, low cytoplasmic binding, and high fluorescent yield. To obtain conjugates of well-defined structure, we sought, for the nonfluorescent backbone, not only a molecule of known structure but one with few reactive sites, preferably only one. Thus, the primary amine group of the synthetic and natural peptides listed in Table 1 was coupled with the fluorescent dyes fluorescein isothiocyanate (FITC), dansyl chloride (DANS), or lissamine rhodamine B

(LRB) (*5*). The conjugation reactions were carried out at room temperature in an aqueous-acetone solvent made alkaline with KHCO$_3$. The products were purified by ion-exchange and gel-permeation chromatography. The criterion for purity was the formation of a single fluorescent spot in paper electrophoresis. Amino acid analysis and end-group analysis were performed on all peptides, except microperoxidase. The purified compounds were dissolved in water and the *p*H was adjusted to about 7 with KOH or HCl.

The solutions of the fluorescent probes were injected into cells of isolated *Chironomus* salivary glands (mid-fourth instar) with the aid of a micropipette and a pneumatic pressure system (*6*); the spread of the fluorescence inside the cells (excited with wavelengths of 460 nm for FITC, 340 nm for DANS, and 540 nm for LRB) was observed and photographed in a microscope darkfield, or, for velocity determinations, the spread was viewed and videotaped with the aid of an image intensifier-television system (*7*). The fluorescent emissions of FITC and DANS peak at 520 and 525 nm (yellow-green) and that of LRB, at 590 nm (red). Thus, in experiments where two tracers were injected together, the LRB was easily distinguished from either of the other two by the use of appropriate filters. In some cases, the tracer studies were combined with measurement of electrical coupling. Electrical current was then passed between the exterior and the interior of the cell injected with the tracer, and the resulting changes in membrane potential were measured in this cell and the immediate neighbor with the use of three microelectrodes (*1*).

The results obtained with the various tracers are summarized in Table 1. The amino acids and peptides with sizes less than or equal to 1158 daltons passed through the junction. Their fluorescence spread from the site of injection throughout the injected cell and into the cell neighbors at rates inversely related to molecular size. The tracers generally crossed several cell junctions on either side. The arrival of a tracer at the junction was marked by an abrupt change in the velocity of the fluorescence spread. With molecules less than or equal to 380 daltons, passage through the first junction became detectable within a few seconds of the tracer's arrival at the junction; and the fluorescence on the two sides of the junction appeared to equalize within 1 to 10 minutes. With molecules between 593 and 1158 daltons, the transit through junction was slower (tak-

Effects of Adenosine Agonists on Intraocular Pressure and Aqueous Humor Dynamics in Cynomolgus Monkeys

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The effects of single or multiple topical doses of the relatively selective A_1 adenosine receptor agonists (R)-phenylisopropyladenosine (R-PIA) and N^6-cyclohexyladenosine (CHA) on intraocular pressure (IOP), aqueous humor flow (AHF) and outflow facility were investigated in ocular normotensive cynomolgus monkeys. IOP and AHF were determined, under ketamine anesthesia, by Goldmann applanation tonometry and fluorophotometry, respectively. Total outflow facility was determined by anterior chamber perfusion under pentobarbital anesthesia. A single unilateral topical application of R-PIA (20–250 μg) or CHA (20–500 μg) produced ocular hypertension (maximum rise = 4·9 or 3·5 mmHg) within 30 min, followed by ocular hypotension (maximum fall = 2·1 or 3·6 mmHg) from 2–6 hr. The relatively selective adenosine A_2 antagonist 3.7-dimethyl-1-propargylxanthine (DMPX, 320 μg) inhibited the early hypertension, without influencing the hypotension. Neither 100 μg R-PIA nor 500 μg CHA clearly altered AHF. Total outflow facility was increased by 71% 3 hr after 100 μg R-PIA. In conclusion, the early ocular hypertension produced by topical adenosine agonists in cynomolgus monkeys is associated with the activation of adenosine A_2 receptors, while the subsequent hypotension appears to be mediated by adenosine A_1 receptors and results primarily from increased outflow facility.

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Key words: adenosine; agonists; antagonists; aqueous; facility; hypertension; hypotension; monkeys; ocular; outflow; pressure; receptors.

1. Introduction

A single topical dose of the relatively A_1 selective adenosine agonists R-PIA and CHA reportedly affected IOP biphasically in rabbits, with ocular hypertension at 30 min followed by pronounced IOP reduction (Camras et al., 1994; Crosson 1992; Crosson and Gray, 1994a, b; Crosson 1995). The physiological mechanism for the ocular hypertension was not clear, but it was inhibited by the adenosine A_2 antagonist DMPX without influencing the IOP reduction. The ocular hypotension was inhibited by the A_1 antagonist 8-cyclopentyl-1,3-dimethylxanthine (CPT) but not by the cyclooxygenase inhibitor indomethacin, suggesting that the hypotension was mediated by adenosine A_1 receptors rather than by production of prostaglandins.

The anatomy and physiology of the aqueous humor formation and drainage apparatus in primates and rabbits differ in important respects (Poyer, Gabelt and Kaufman, 1992), the effect of adenosine agonists and antagonists on IOP and aqueous humor dynamics has not been studied in primates, and the hypotensive response could be associated with any one or more of the four parameters that affect IOP (decreased AHF, increased outflow facility, increased uveoscleral outflow and decreased episcleral venous pressure). We report here the effects of single or multiple doses of R-PIA or CHA on aqueous humor dynamics in normotensive cynomolgus monkey eyes in vivo.

2. Materials and Methods

Animals

Cynomolgus monkeys (*Macaca fascicularis*), weighing 1·6–5·6 kg, were studied. All investigations were in accordance with University of Wisconsin and NIH guidelines, and with the ARVO Statement on the Use of Animals in Ophthalmic and Vision Research.

Anesthesia

Anesthesia for IOP and AHF measurements was induced with i.m. ketamine (10 mg kg⁻¹) and maintained with supplemental i.m. injections as required (usually 5 mg kg⁻¹ every 30–45 min). Anesthesia for total outflow facility was induced by i.m. ketamine (10 mg kg⁻¹) followed by i.m. pentobarbital-Na (35 mg kg⁻¹).

Drug Preparation and Administration

R-PIA, CHA and DMPX were obtained from Research Biochemical Inc., Natick, MA, U.S.A., and dimethyl sulfoxide (DMSO) from Research Industries Corporation, Salt Lake City, UT, U.S.A. R-PIA, CHA and DMPX solutions were prepared in DMSO and

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water as follows: the 20 μg and 100 μg doses of R-PIA or CHA were dissolved in 10 μl of 15% DMSO; 250 μg of R-PIA was dissolved in 25 μl of 50% DMSO; 500 μg of CHA was dissolved in 20 μl of 25% DMSO; 160 μg of DMPX was dissolved in 32 μl of 20% DMSO. Vehicle was the corresponding concentration of DMSO used to prepare the drug solutions. R-PIA, CHA or their vehicles were administered in 5 μl drops. DMPX or vehicle was administered in 8 μl drops.

For IOP and AHF studies, drugs were administered topically to the central cornea of the supine monkey at 30 sec intervals, with blinking prevented between each drop. For single dose experiments or on days 1 and 4 in multiple dose experiments, drugs or vehicles were administered under ketamine anesthesia. On days 2 and 3 in multiple dose experiments, the drugs or vehicles were administered twice daily (at 09:00 and 15:00) to fully conscious manually restrained monkeys.

Intraocular Pressure Measurement

IOP was determined with a 'minified' Goldmann applanation tonometer (Kaufman and Davis, 1980), using 'Half and Half®' creamer solution (Borden Inc. Columbus, OH, U.S.A.) as the tear film indicator, with the monkey lying prone in a head holder and the eyes positioned at 4 to 8 cm above the heart. For each eye, three IOP readings taken 5–10 min apart were averaged as a baseline before administration of drugs and vehicles. Single IOP readings were taken at 0·5, 1, 2, 3, 4, 5 and 6 hr after 20–250 μg R-PIA or 20–500 μg CHA for the single dose protocol; at 0·5, 2, 4, 6 and 9 hr on day 1 and 0·5, 1, 2, 4, 6 and 9 hr on day 4 for the multiple treatment protocol; at 0·5, 1, 2, 3, 4, 5, and 6 hr after 100 μg R-PIA or 500 μg CHA for the bilateral agonist and unilateral antagonist protocol (160 μg DMPX was administered 30 and 60 min before agonist); or at 1, 2, 3, 4, 5, 6, and 7 hr after the first dose of DMPX for the antagonist-only protocol. At least one week elapsed between IOP experiments in a given animal.

Aqueous Humor Flow Measurement

At 1700 hr, background fluorophotometric readings were taken. Immediately thereafter, one drop of 0·5% proparacaine hydrochloride (Alcaine®, Alcon Laboratories, Ft. Worth, TX, U.S.A.) was administered to the central cornea of both eyes of each supine monkey (to enhance corneal penetration of fluorescein), followed 5 min later by four 2 μl drops of 2% fluorescein-Na (Alcon) at 30 sec intervals. Drugs or vehicles were administered at 0900–1000 hr the next day. Corneal and anterior chamber fluorescence were measured using a Coherent Fluorotron Master® scanning ocular fluorophotometer. The average of two scans was taken for each eye every 30 minutes for 6 hr after agonist administration for all protocols. In the agonist-only

protocol, no AHF baseline was measured. During the R-PIA experiment, AHF in three monkeys was measured for only 3 hr due to subsequent equipment problems. In the bilateral agonist and unilateral antagonist protocol, baseline AHF was determined 1 week before and 7 weeks after bilateral 500 μg CHA and unilateral 320 μg DMPX administration (160 μg DMPX was administered 30 and 60 min before 500 μg CHA), with no experiments performed before the first baseline or between drug administration and the second baseline. No significant ipsilateral or contralateral differences were observed within or between the two baselines, so the average of the two baselines within the corresponding time periods was used. In all other protocols, at least 4 weeks elapsed between AHF experiments in a given animal. AHF was calculated from the scans by a modified method of Jones and Maurice (Jones and Maurice, 1966; Gabelt et al., 1995).

Outflow Facility Measurement

Total outflow facility was determined by 2-level constant pressure perfusion of the anterior chamber with Bárány's mock aqueous humor (Bárány, 1963), using a one-needle technique and correcting for internal apparatus resistance (Bárány, 1965). Most monkeys had undergone more than one prior anterior chamber perfusion, but none within the preceding 5–6 weeks. All had biomicroscopically normal anterior segments, free of cells and flare, at the time of the present experiments. Baseline facility in both eyes was determined for 35 min. This was followed by topical administration of 100 μg of R-PIA to the superior cornea of the treated eye and 15% DMSO to the control eye. The lower eyelid was lifted several times immediately after each drop to ensure good drug/vehicle contact with the cornea. The reservoirs were closed for 3 hr after drug administration to minimize resistance washout (Kaufman, True-Gabelt and Erickson-Lamy, 1988) and then opened for post-drug outflow facility determination for 35 min.

Slit Lamp Examination

Slit lamp biomicroscopy was performed by a trained ophthalmologist before drug administration, during single-dose IOP experiments at 3 and 6 hr after dosing, and during multiple dose experiments at 2, 6 and 9 hr after the first dose and at 2, 6, 9, and 24 hr after the seventh dose. The integrity of the corneal epithelium, and the presence or absence of flare (protein) in the anterior chamber, an indication of blood-aqueous barrier breakdown, were noted.

Data Analysis

Data are presented as mean ± S.E.M. for n eyes or animals. Pre- or post-drug treated vs. contralateral

control; post-drug or post-vehicle vs. ipsilateral base-line; and baseline corrected post-drug treated vs. control comparisons were made, using the 2-tailed paired t-test for ratios vs. 1·0 or differences vs. 0·0. Comparisons between monkey ages in different treatment groups were made, using the 2-tailed unpaired t-test for differences and the χ^2-test for age distribution (< 3 yr vs. ⩾ 3 yr).

3. Results

Intraocular Pressure

The following text refers to baseline- and control eye-adjusted IOP data unless otherwise stated; the absolute and adjusted data are given in Figs 1–5. A single dose of R-PIA or CHA administered unilaterally produced a biphasic effect on IOP: early ocular hypertension at 30 min, followed by ocular hypotension from 2–6 hr. With R-PIA, maximum hypertension of 4·86±0·84 mmHg was produced with the 250 μg dose; maximum hypotension of 2·11±0·38 mmHg occurred 4 hr after the 100 μg dose (Fig. 1). With CHA, maximum hypertension of 3·50±1·53 mmHg and hypotension of 3·60± 0·73 mmHg (at 4 hr) were produced with the 500 μg dose (Fig. 2).

On day 4 of twice daily treatment with 100 μg of R-PIA (Fig. 3) or 500 μg of CHA (Fig. 4), neither the maximum hypertension (at 30 min) nor the maximum subsequent hypotension, adjusted for baseline and control eye values, were significantly different from that on day 1. However, both drugs tended to enhance the time-dependent IOP decrease in the control eyes. The duration of ipsilateral IOP reduction was longer in the treated eyes for both agonists on day 4 than on day
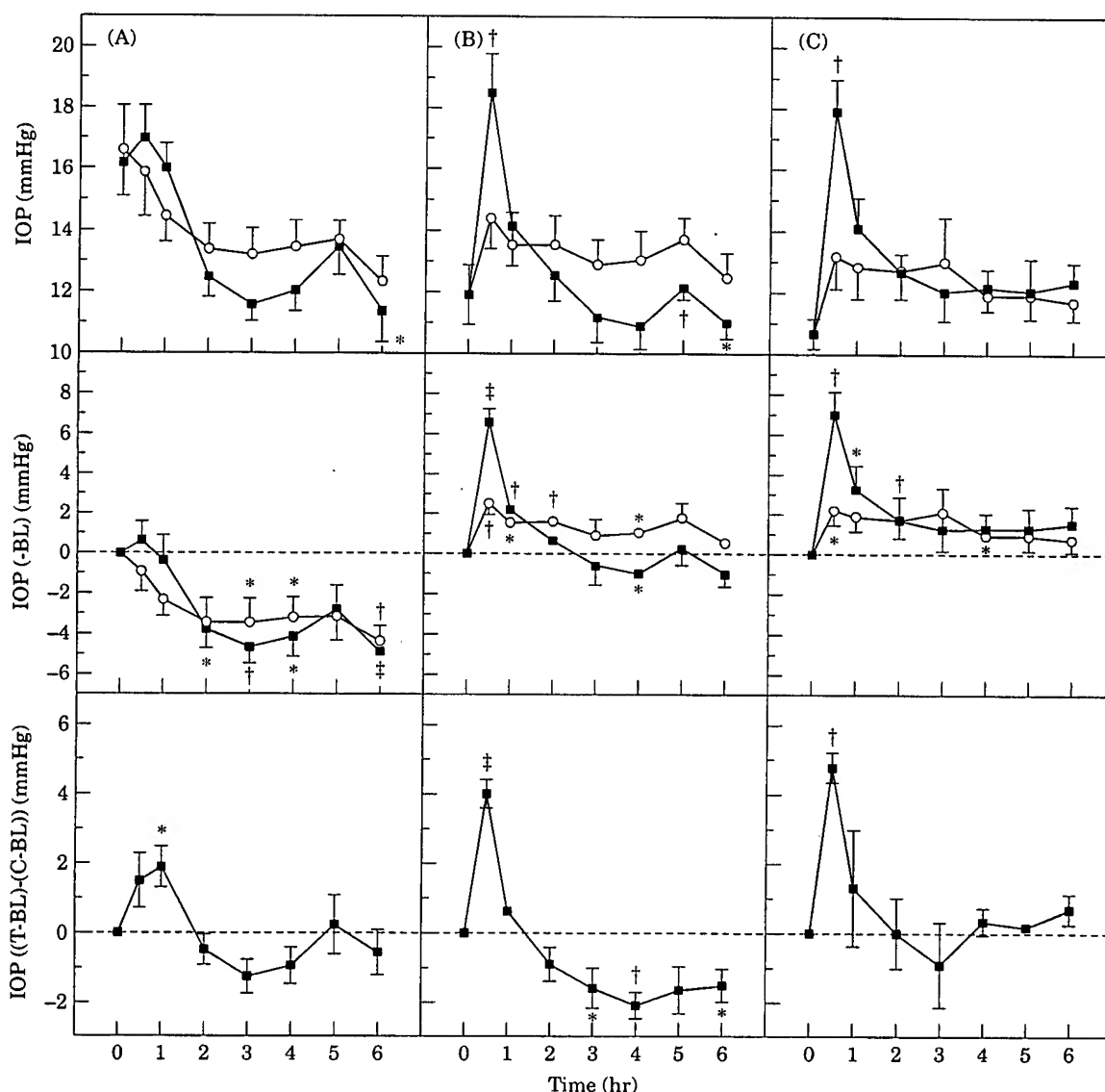

FIG. 1. Effect of a single topical dose of R-PIA on IOP in cynomolgus monkeys. (A) 20 μg R-PIA to treated eye; 15% DMSO to control eye (5 μl × 2, n = 5). (B) 100 μg R-PIA to treated eye; 15% DMSO to control eye (5 μl × 2, n = 6). (C) 250 μg R-PIA to treated eye; 50% DMSO to control eye (5 μl × 5, n = 6). IOP data are mean ±s.e.m. mmHg. IOP difference between eyes (1st row), from baseline (2nd row) or between eyes corrected for baseline (3rd row) were tested for differences ≠ 0·0 by the 2-tailed paired t-test: *P < 0·05; †P < 0·01; ‡P < 0·001. ■, R-PIA; ○, vehicle.
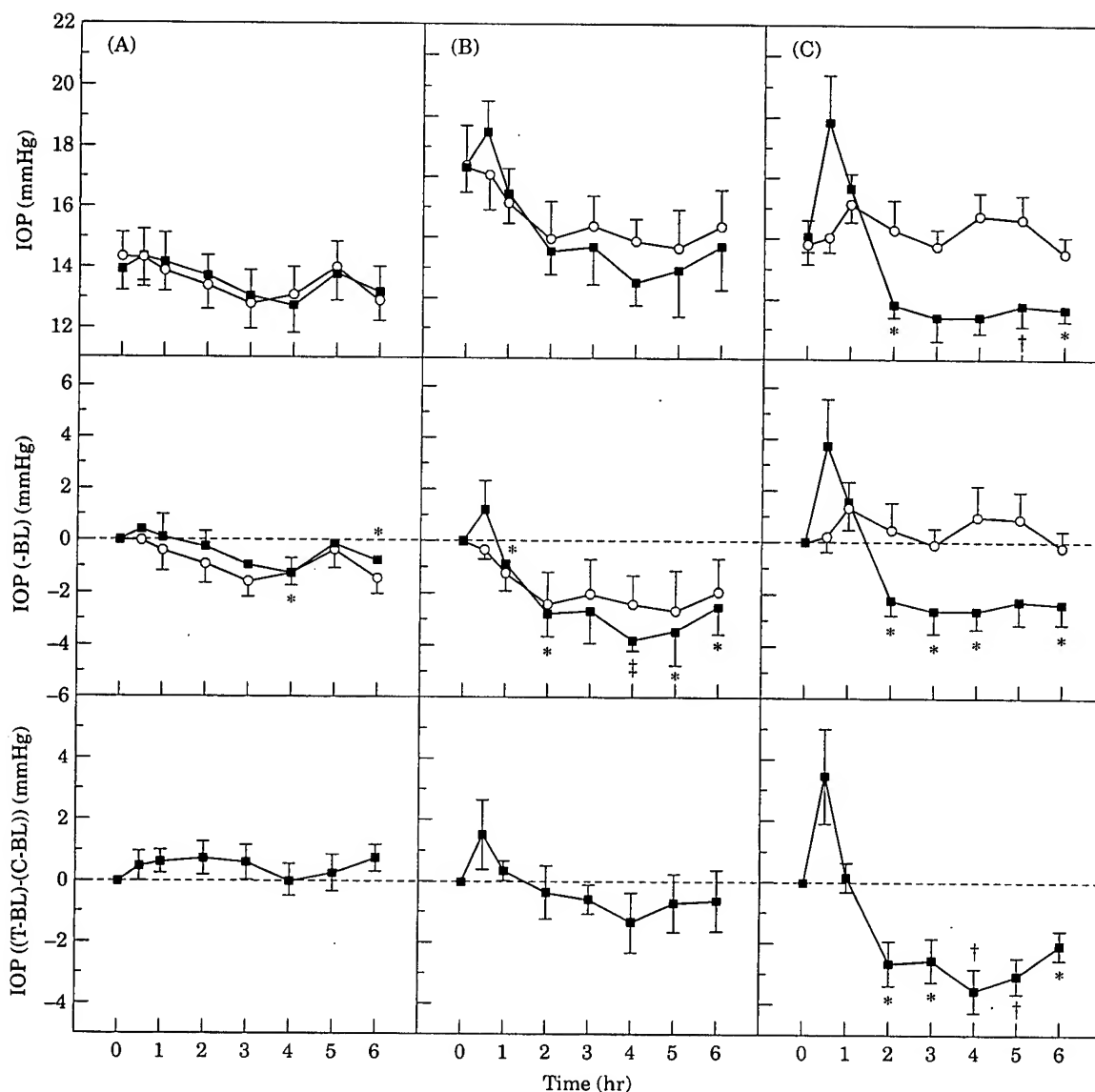
FIG. 2. Effect of a single topical dose of CHA on IOP in cynomolgus monkeys. (A) 20 μg CHA to treated eye; 15% DMSO to control eye (5 μl × 2, n = 5). (B) 100 μg CHA to treated eye; 15% DMSO to control eye (5 μl × 2, n = 7). (C) 500 μg CHA to treated eye; 25% DMSO to control eye (5 μl × 4, n = 5). IOP data are mean ± S.E.M. mmHg. IOP difference between eyes (1st row), from baseline (2nd row) or between eyes corrected for baseline (3rd row) were tested for differences ≠ 0·0 by the 2-tailed paired t-test: *$P < 0.05$; †$P < 0.01$; ‡$P < 0.001$. ■, CHA; ○, vehicle.

1, compared to same-day ipsilateral baseline; compared to baseline- and control eye-corrected values, day 4 duration exceeded day 1 duration for R-PIA but not CHA. The maximum hypotension in R-PIA-treated eyes compared to baseline was greater in magnitude on day 4 than on day 1; IOP in the contralateral control eyes was also reduced significantly on day 4, but the effect was smaller in magnitude than in drug-treated eyes.

In bilateral agonist + unilateral antagonist experiments, pretreatment with the A_2 antagonist DMPX (320 μg) substantially attenuated the initial agonist-induced hypertension at 0·5 hr. The IOP at 0·5 hr in the agonist + antagonist-treated eye was significantly lower than in the contralateral agonist-only treated eye (1·69 ± 1·07 mmHg vs. 5·06 ± 0·70 mmHg in R-PIA–DMPX protocol; 1·97 ± 0·59 mmHg vs.

4·81 ± 0·93 mmHg in CHA–DMPX protocol; $P < 0.01$). IOP in the DMPX-pre-treated eye tended to remain lower (albeit not significantly) throughout the experiment. DMPX alone had no significant effect on IOP (Fig. 5).

Aqueous Humor Flow

AHF did not generally differ significantly between treated and control eyes following either R-PIA or CHA (Table I). In the bilateral agonist–unilateral antagonist experiment, AHF was decreased by 24–31% ($P < 0.05$) in the DMPX + CHA-treated eyes and by 9–15% ($P > 0.05$) in the contralateral CHA only-treated eyes, compared to no-drug baselines. AHF in the DMPX + CHA-treated eyes generally averaged ~ 10% less than in contralateral CHA only-treated
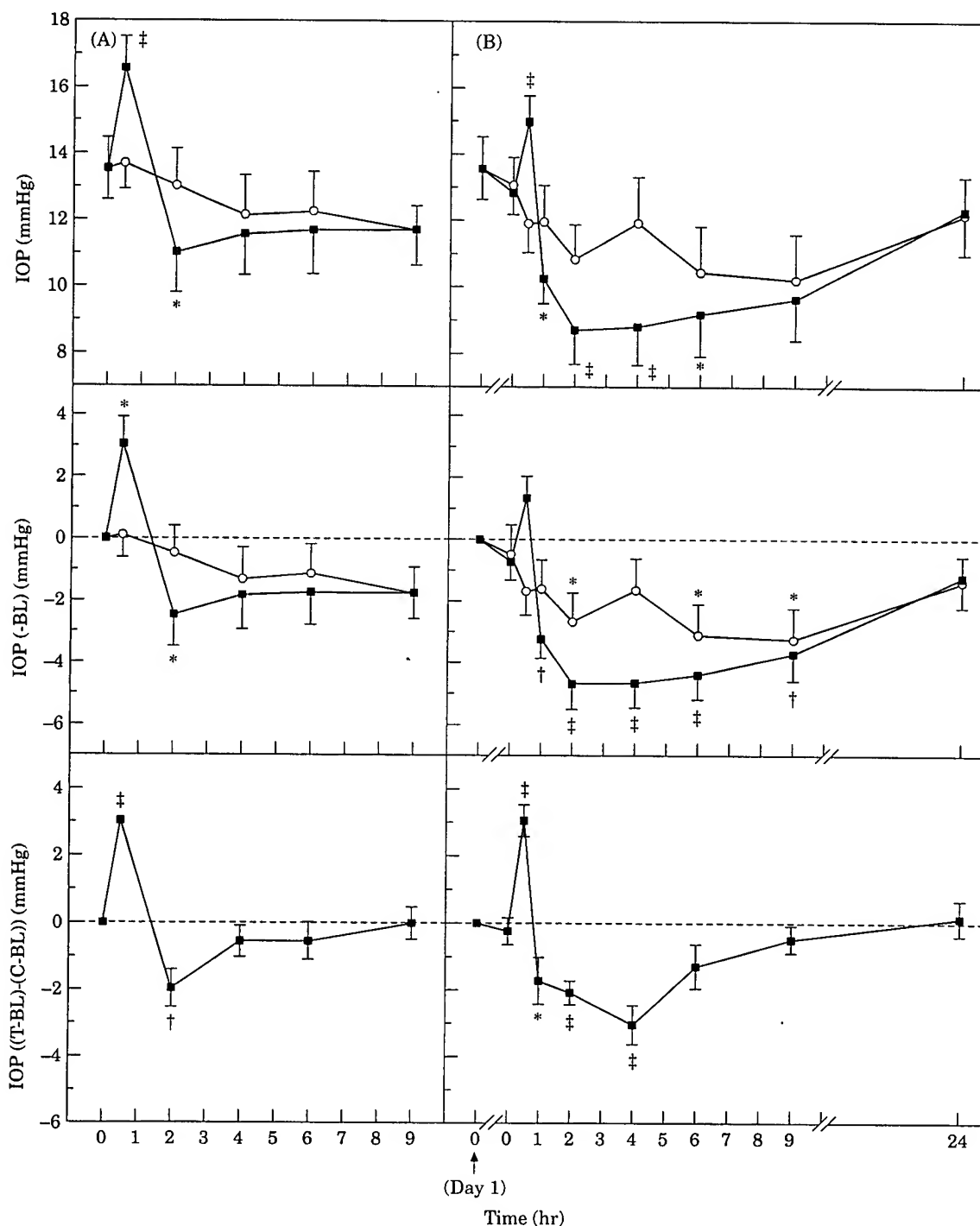
Fig. 3. Effect of twice daily topical (100 μg × 7) R-PIA doses on IOP in cynomolgus monkeys n = 9. (A) Day 1: one dose of 100 μg R-PIA (5 μl × 2) to treated eye; 15% DMSO (5 μl × 2) to control eye; (B) Day 4: seventh dose of 100 μg R-PIA (5 μl × 2) to treated eye; 15% DMSO (5 μl × 2) to control eye (twice daily). IOP data are mean ± s.e.m. mmHg. IOP difference between eyes (1st row), from baseline (2nd row) or between eyes corrected for baseline (3rd row) were tested for differences ≠ 0·0 by the 2-tailed paired t-test: *P < 0·05; †P < 0·01; ‡P < 0·001. ■, R-PIA; ○, vehicle.

eyes, whether or not adjusted for the respective no-drug baselines, but the ratio did not differ significantly from 1·0 (P > 0·05) over any time interval (Table II).

Outflow Facility

Topical administration of 100 μg of R-PIA increased outflow facility relative to baseline by 71% compared to contralateral control eyes (corrected for perfusion-

induced resistance washout, as estimated from the post-drug/pre-drug facility ratio in the control eyes) at 3–3·5 hr (Table III).

Slit Lamp Examination

Slit lamp biomicroscopy 3 hr after a single topical dose of CHA (100 μg) revealed very mild flare (1 + on a scale of 4) in the anterior chambers of the treated
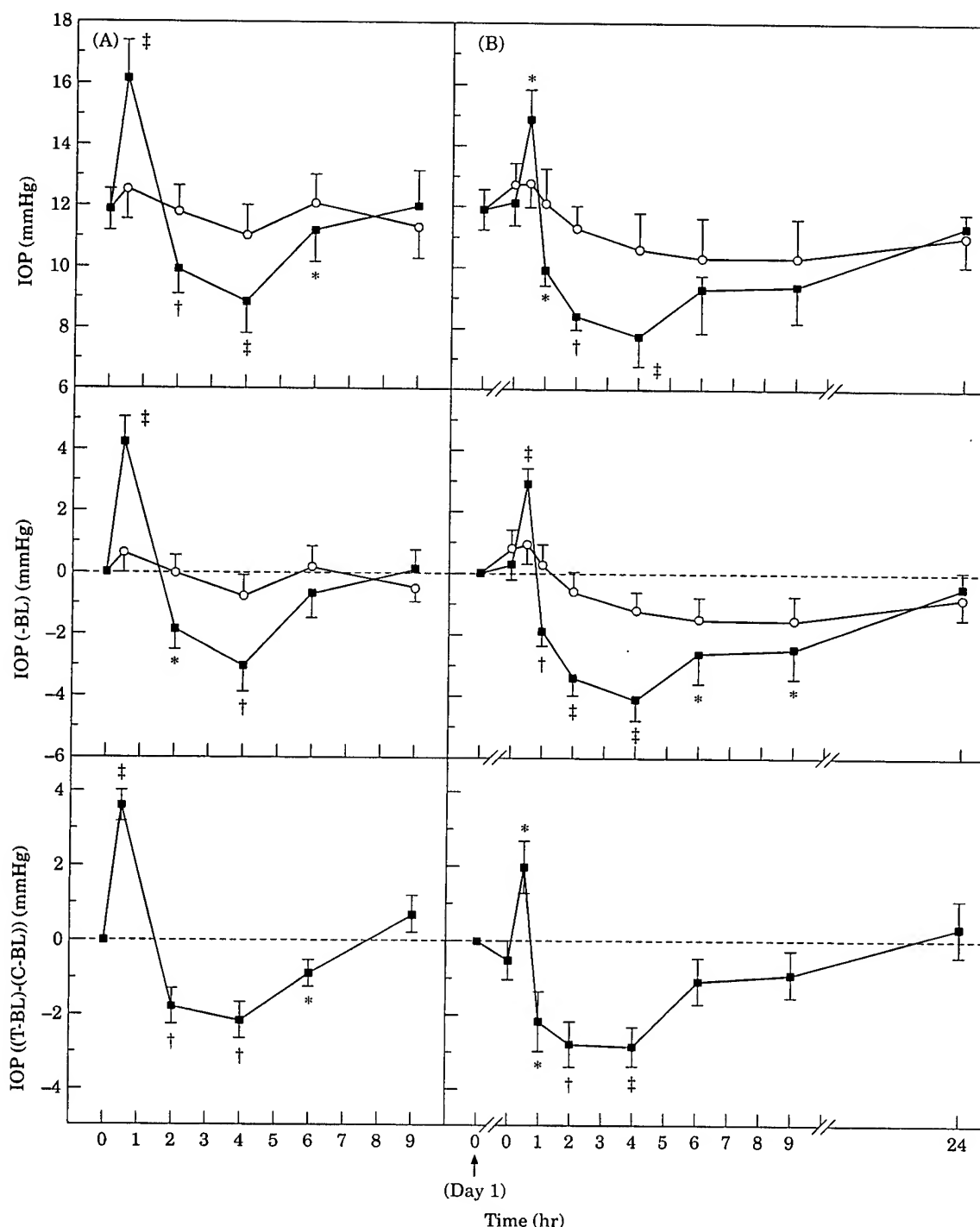
FIG. 4. Effect of twice daily topical (500 μg × 7) CHA doses on IOP in cynomolgus monkeys n = 9. (A) Day 1: one dose of 500 μg CHA (5 μl × 4) to treated eye; 25% DMSO (5 μl × 4) to control eye; (B) Day 4: seventh dose of 500 μg CHA (5 μl × 4) to treated eye; 25% DMSO (5 μl × 4) to control eye (twice daily). IOP data are mean ± S.E.M. mmHg. IOP difference between eyes (1st row), from baseline (2nd row) or between eyes corrected for baseline (3rd row) were tested for differences ± 0·0 by the 2-tailed paired t-test: *P < 0·05; †P < 0·01; ‡P < 0·001. ■, CHA; O, vehicle.

and control eyes of 1 out of 7 monkeys, which disappeared (treated eye) or decreased significantly (control eye) by 6 hr. Slit lamp examination also revealed 1+ flare following 250 μg R-PIA in 3 of 5 treated eyes, which disappeared completely by 6 hr. Mild superficial punctate corneal epitheliopathy was observed bilaterally during all single or multiple topical treatment experiments, as is typical with repeated

tonometry. There were no apparent differences between treated and control eyes, and between single and multiple treatments. No abnormal findings, except the bilateral punctate keratopathy were observed during the DMPX/agonists or DMPX alone IOP experiments. The presence or absence of external ocular vasodilation was difficult to discern in the heavily pigmented monkey conjunctiva.
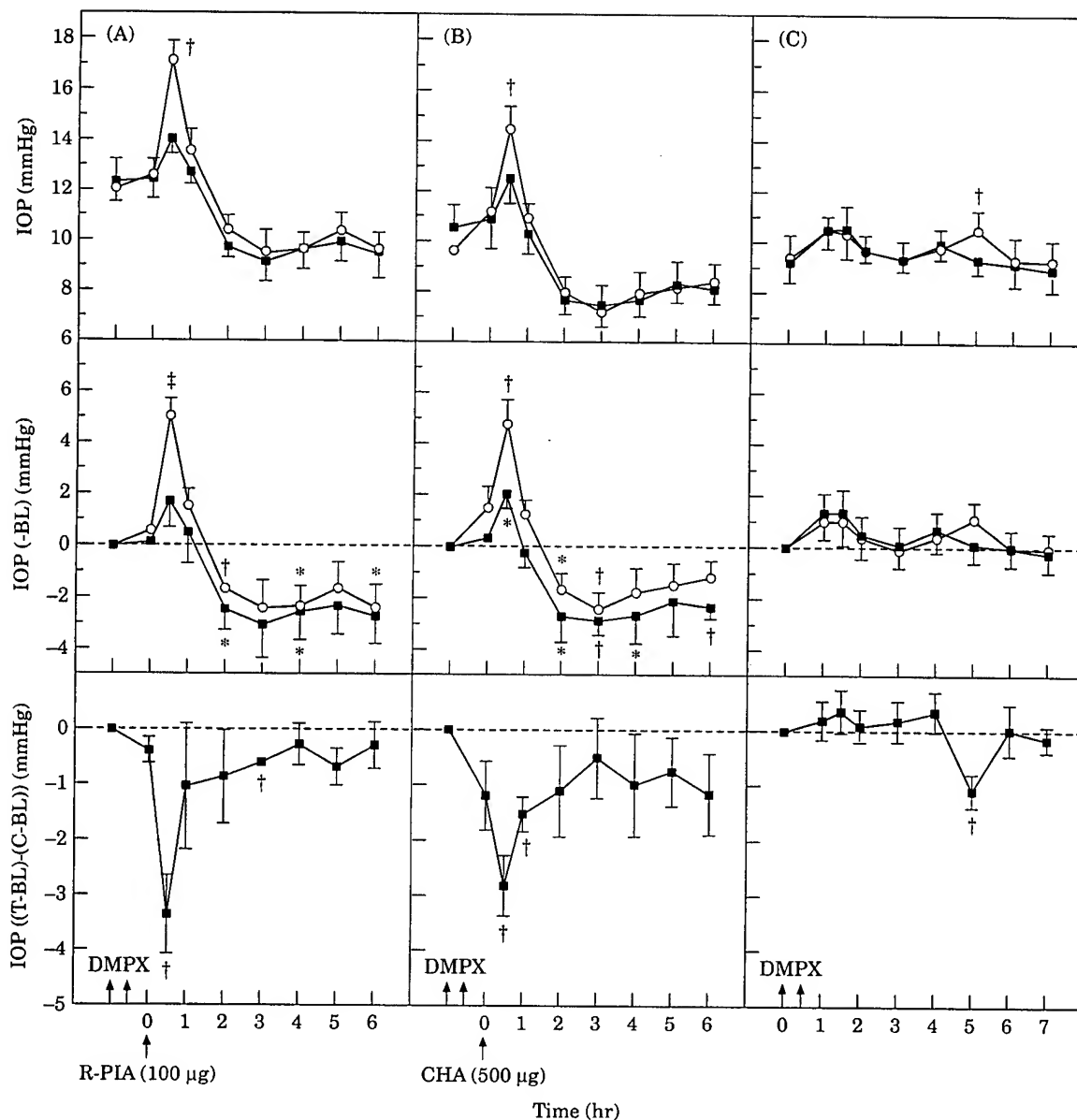
FIG. 5. Effect of two topical (160 μg × 2) DMPX doses on IOP in cynomolgus monkeys $n = 6$. (A) 160 μg DMPX (8 μl × 4) to treated eye 30 and 60 min before 100 μg R-PIA (5 μl × 2); 20% DMSO (8 μl × 4) to control eye 30 and 60 min before 100 μg R-PIA (5 μl × 2); ■, DMPX + R-PIA, ○, R-PIA. (B) 160 μg DMPX (8 μl × 4) to treated eye 30 and 60 min before 500 μg CHA (5 μl × 4); 20% DMSO (8 μl × 4) to control eye 30 and 60 min before 500 μg CHA (5 μl × 4); ■, DMPX + CHA; ○, CHA. (C) 160 μg DMPX (8 μl × 4) to one eye at hr 0 and hr 0·5; 20% DMSO (8 μl × 4) to opposite eye at hr 0 and hr 0·5; ■, DMPX; ○, vehicle. IOP data are mean ± S.E.M. mmHg. IOP difference between eyes (1st row), from baseline (2nd row) or between eyes corrected for baseline (3rd row) were tested for differences ≠ 0·0 by the 2-tailed paired t-test: *$P < 0·05$; †$P < 0·01$; ‡$P < 0·001$.

4. Discussion

The IOP response to the adenosine agonists R-PIA and CHA in cynomolgus monkeys, reported for the first time in this study, was generally similar to that previously reported in rabbits (Camras et al., 1994; Crosson, 1992, 1995). In rabbits treated topically with R-PIA (Crosson, 1992), ocular hypertension of 3·5 mmHg at 0·5 hr was found after a 500 μg dose and was followed by a significant IOP reduction (5–8 mmHg), compared to vehicle-treated rabbits. At lower R-PIA doses, Crosson's rabbits exhibited no ocular hypertension but the reduction of IOP was still significant. Camras et al. (1994) administered 200 μg

of R-PIA topically to rabbits and produced an 8·4 mmHg IOP rise at 0·5 hr and a maximum IOP reduction of 5·8 mmHg at 5 hr, compared with contralateral control eyes. In rabbits treated topically with CHA (Crosson, 1995), an initial hypertensive response of 2·3 ± 0·8 mmHg at 0·5 hr was induced after the administration of the 500 μg dose, followed by a significant reduction in IOP of 4–6 mmHg from 2–6 hr. In our monkeys, both R-PIA and CHA had a biphasic effect on IOP, producing ocular hypertension of ～4 mmHg within 30 min, followed by an IOP reduction of 2–3·5 mmHg from 2–6 hr with a single dose of 100 μg R-PIA or 500 μg CHA.

Although R-PIA and CHA are relatively selective

TABLE I

Aqueous flow after topical 100 μg R-PIA or 500 μg CHA in monkeys

	Hours 0·5–1·5			Hours 0·5–3·0		
	Trt	Cont	T/C	Trt	Cont	T/C
R-PIA	1·25±0·12	1·15±0·13	1·15±0·12	1·32±0·14	1·16±0·13	1·16±0·06*
CHA	0·76±0·14	0·88±0·10	0·88±0·14	1·08±0·13	1·02±0·16	11·12±0·12

	Hours 3·5–6·0			Hours 0·5–6·0		
	Trt	Cont	T/C	Trt	Cont	T/C
R-PIA	1·37±0·29	1·25±0·19	1·07±0·10	1·41±0·27	1·32±0·21	1·05±0·08
CHA	1·36±0·25	1·42±0·22	0·94±0·04	1·28±0·19	1·25±0·17	1·02±0·04

Topical administration of 100 μg R-PIA or 500 μg CHA in treated eyes and vehicle in control eyes. Flow data are mean±s.e.m. μl min⁻¹, with R-PIA. $n = 8$ during hr 0·5–3·0, $n = 5$ during hr 3·5–6·0; With CHA. $n = 5$; *$P < 0·05$ by the 2-tailed paired t-test for ratios ≠ 1·0. Times are hr post-treatment.

TABLE II

Aqueous flow after topical 500 μg CHA or 320 μg DMPX + CHA in monkeys

	Hours 0·5–1·5			Hours 0·5–3·0		
	Trt	Cont	T/C	Trt	Cont	T/C
BL	1·32±0·15	1·17±0·15	1·17±0·12	1·41±0·15	1·37±0·18	1·05±0·05
Exp	0·85±0·08	1·01±0·15	0·99±0·24	1·03±0·09	1·13±0·12	0·92±0·05
Exp/BL	0·69±0·11*	0·88±0·11	0·90±0·26	0·75±0·06†	0·85±0·06	0·89±0·05

	Hours 3·5–6·0			Hours 0·5–6·0		
	Trt	Cont	T/C	Trt	Cont	T/C
BL	1·58±0·15	1·63±0·25	1·07±0·17	1·60±0·16	1·56±0·21	1·06±0·07
Exp	1·20±0·14	1·38±0·14	0·87±0·06	1·16±0·10	1·28±0·12	0·92±0·04
Exp/BL	0·76±0·03†	0·91±0·10	0·88±0·10	0·73±0·03†	0·86±0·08	0·88±0·05

Topical administration of 320 μg DMPX and 500 μg CHA in treated eyes and 500 μg CHA in control eyes. BL. baseline flow (average of two separate baselines one week before and seven weeks after drugs); Exp. flow after drugs. Flow data are mean±s.e.m. μl min⁻¹. $n = 6$; *$P < 0·05$; †$P < 0·01$ by the 2-tailed paired t-test for ratios ≠ 1·0. Times are hr post-treatment.

TABLE III

Effect of a single topical 100 μg R-PIA dose on outflow facility

	Outflow facility			
	Treated	Control	T-C	T/C
BL	0·336±0·070	0·369±0·080	−0·033±0·058	1·023±0·122
R-PIA	0·628±0·163	0·398±0·091	0·230±0·083*	1·644±0·162†
R-PIA/BL	1·839±0·143	1·129±0·100	0·710±0·157†	1·712±0·183†

Facility data are mean±s.e.m. (μl min⁻¹ mmHg⁻¹); $n = 9$; BL = baseline. Ratio ≠ 1·0. difference ≠ 0·0 by the 2-tailed paired t-test: *$P < 0·05$. †$P < 0·01$.

adenosine A_1 agonists (Bruns, Lu and Pugsley, 1986), studies in rabbits have demonstrated that at the topical doses used in this study these agents can activate both adenosine A_1 and A_2 receptors in the eye (Crosson, 1992, 1995; Crosson and Gray, 1996). In both Crosson and Gray's, (1996) rabbit study and our monkey study, pretreatment with the adenosine A_2 antagonist DMPX substantially blocked the early
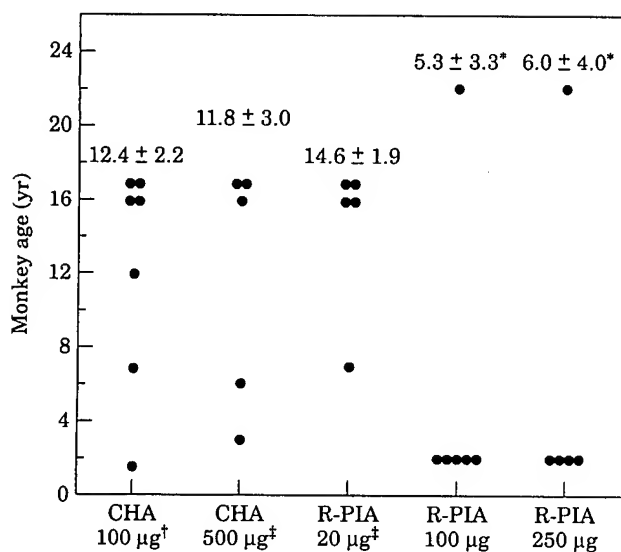
FIG. 6. Monkey ages in different treatment groups. Each point represents one animal. Group data are mean ± S.E.M. yr. Age data for the 20 μg CHA group and for one monkey in the 250 μg R-PIA group not available. *$P < 0.001$ by the 2-tailed unpaired t-test for age differences between 100 μg R-PIA or 250 μg R-PIA group and 20 μg R-PIA, 100 μg CHA or 500 μg CHA group. †$P < 0.0$, ‡$P < 0.025$ by the χ^2-test for age distribution (< 3 yr vs. $\geqslant 3$ yr) between 100 μg CHA, 500 μg CHA or 20 μg R-PIA group and 100 μg R-PIA or 250 μg R-PIA group.

hypertension without attenuating the subsequent hypotension, indicating that the hypertensive phase is associated with A_2 receptor stimulation. In our monkeys, IOP in the DMPX-pre-treated eye tended to remain lower throughout the experiment, suggesting that A_2 receptor stimulation may not only cause the initial hypertension, but also induce a functional antagonism of the A_1 receptor-mediated hypotension similar to that described in rabbits (Crosson, 1995). Crosson (1992) also demonstrated that the adenosine A_1 antagonist CPT can inhibit the hypotensive response to R-PIA. An A_1 antagonist was not employed in the current study, but absent attenuation of the hypotension by the A_2 antagonist, it seems reasonable to attribute the hypotension to A_1 receptor stimulation.

R-PIA and CHA are close structural analogs exhibiting similar pharmacokinetic profiles and approximately equal activity at the receptors (Crosson, 1992, 1995). In Crosson's rabbits, the hypertensive and hypotensive responses to both R-PIA and CHA were dose-dependent. In our monkeys, the hyper- and hypotensive responses to CHA both tended to be dose-dependent. However, in animals treated with R-PIA dose-dependence was observed only for the early rise in IOP. Although significant IOP reduction occurred with the mid-range dose of R-PIA (100 μg), a hypotensive response was not evident for the highest R-PIA dose (250 μg) evaluated, perhaps related to the age of the monkeys. Monkeys receiving 100 μg and 250 μg of R-PIA were primarily 2-year olds and much younger than those in the other groups (Fig. 6). Such young monkeys usually have lower baseline IOP (Fig.

1), so that outflow facility enhancement or AHF reduction will have less IOP effect than at higher starting IOP (Kaufman, 1985). Additionally, the levels of adenosine A_2 receptor mRNA and adenosine A_2 binding sites are reduced by 32% and 20% respectively in old compared to young rat striatum (Schiffmann and Vanderhaeghen, 1993), suggesting that the density of adenosine A_2 receptors is higher in younger animals. This conclusion appears to be consistent with the data presented in Figs 1 and 2 where the hypertensive response to the younger monkeys treated with R-PIA is much larger than the corresponding response to CHA in older animals. In rabbits and cats of equal age no difference in hypertensive response is observed when CHA and R-PIA are compared (Crosson, unpublished results). Since the net change in IOP likely results from a combination of the hypertensive and hypotensive responses, a larger hypertensive response to 100 or 250 μg R-PIA in the two younger animal groups may have blunted the subsequent IOP reduction and obscured its dose-dependence (Fig. 1).

In our study, no significant differences were observed between the hypertensive responses to either agonist on day 4 and day 1. The ipsilateral hypotensive response following 100 μg R-PIA increased in magnitude on day 4 compared to day 1. However, the control eyes also exhibited a hypotensive response on day 4, reducing the apparent magnitude and duration of the baseline- and control eye-adjusted change. The IOP reduction in the contralateral control eyes was greater on day 4 than day 1, suggesting it was not due to ketamine anesthesia alone (Hahnenberger, 1976; Gabelt et al., 1994) but perhaps to a contralateral response to the A_1 effect of the adenosine agonists (Crosson, 1995).

Investigation of the physiological mechanism of the adenosine agonist-induced ocular hypertension or hypotension in rabbits has yielded conflicting results. Camras et al. (1994) found a transient increase in AHF by fluorophotometry during the hypertensive phase following a single 200 μg dose of R-PIA, but no effect on AHF during the hypotensive phase compared to contralateral control eyes. Crosson and Gray (1996) demonstrated that topical administration of 165 μg of the adenosine A_2 agonist CV-1808 was associated with increased aqueous humor flow and protein concentration. Braunagel, Xiao and Chiou (1988) confirmed that adenosine produced retinal and choroidal vasodilatation in rabbits. Anterior uveal vasodilatation could induce breakdown of the blood-aqueous barrier and consequently increased IOP (Funk, Wagner and Rohen, 1992; Krootila et al. 1992). However, at lower doses of CV-1808 IOP rose without an increase in aqueous humor protein, suggesting that two independent mechanisms are responsible for the adenosine A_2-induced rise in IOP (Crosson and Gray, 1996). In addition, Crosson (1995) demonstrated that R-PIA (165 μg) and CHA (165 μg)

reduced AHF by 37% and 35% respectively during the early part of hypotensive response when compared to other vehicle-treated rabbits.

In our monkeys, there was little evidence for an early increase in AHF after 100 μg R-PIA or 500 μg CHA, and certainly not one sufficient to account for the early ocular hypertension. At high doses of adenosine A_2 agonists the blood-aqueous barrier may break down secondary to hemodynamic changes in the ciliary body, perhaps producing a minimal increase in AHF while at low doses of A_2 agonists or with relatively selective A_1 agonists such as R-PIA or CHA, the transient IOP rise may be associated with vascular expansion in the ciliary body without increased AHF. There was also little evidence for a subsequent decrease in AHF during the hypotensive phase. However, when compared to baselines obtained on separate occasions, AHF was significantly decreased by 24–31% in CHA + DMPX-treated eyes (Table II). It may be that A_1 receptor stimulation slightly decreased ipsilateral AHF but the apparent effect was minimized by (1) a contralateral A_1 receptor-related inhibition of AHF and (2) an ipsilateral A_2 receptor-related effect. Overall these data suggest that in monkeys adenosine agonists at most have only a modest effect on AHF.

In rabbits, total outflow facility (2-level constant pressure perfusion) after 200 μg R-PIA was significantly increased during the hypotensive phase (Camras et al., 1994). Perfusion of the anterior chamber of monkey eyes with drug free Bárány's mock aqueous produces a time-dependent facility increase, presumably due to washout or resistance-producing extracellular material from the trabecular meshwork (Kaufman et al., 1988). In the present experiments, the resistance washout was minimized by closing the reservoirs after baseline facility measurements until beginning post-drug facility measurements. The 71% increase in outflow facility 3–3·5 hr following topical R-PIA (relative to the control eye and adjusted for resistance washout) in our monkeys (Table III) is sufficient to produce the observed IOP reduction, according to the modified Goldmann equation (Kaufman, 1985). Our control eye post-drug/pre-drug facility ratio of 1·13 is consistent with the ~15% 'washout effect' seen in other comparably-timed perfusions without drugs (Kaufman et al., 1988), indicating the absence of a contralateral drug effect on facility, contralateral effects on IOP not withstanding.

We saw little apparent ocular toxicity following topical R-PIA, CHA, DMPX, or DMSO vehicle by slit lamp biomicroscopy. The mild superficial punctate keratopathy was consistent with repeated tonometry. The presence or absence of external ocular vasodilation was not discerned because of the heavily pigmented monkey conjunctiva. Mild transient anterior chamber flare was seen only in some 2 yr-old monkeys, mainly in eyes treated with the higher dose of R-PIA. Such flare indicates breakdown of the blood-aqueous barrier, as in Crosson and Grays (1996)

rabbits. Because the younger monkeys may have more A_2 receptors (Schiffmann and Vanderhaeghen, 1993) whose activation may produce more aqueous humor protein (Crosson and Gray 1996) and resultant ocular hypertension, the biomicroscopic findings support the hypothesis that activation of A_2 receptors may have blunted the subsequent ocular hypotension especially in the younger animals.

In summary, these results demonstrate that relatively selective adenosine A_1 agonists produce a biphasic change in IOP following topical administration to monkeys: early hypertension followed by prolonged hypotension. The hypertensive and hypotensive phases are associated with activation of adenosine A_2 and A_1 receptors respectively. The IOP decrease appears to result primarily from an increase in outflow facility.

Acknowledgements

References

Bárány, E. H. (1963). Simultaneous measurement of changing intraocular pressure and outflow facility in the vervet monkey by constant pressure infusion. *Invest. Ophthalmol.* 3, 135–43.

Bárány, E. H. (1965). Relative importance of autonomic nervous tone and structure as determinants of outflow resistance in normal monkey eyes (*Cercopithecus ethiops* and *Macaca irus*). In (Ed. Rohen, J. W.). *The structure of the eye, Second Symposium*. Pp. 223–36. FK Schattauer Verlag: Stuttgart.

Braunagel, S. C., Xiao, J. G. and Chiou, G. C. Y. (1988). The potential role of adenosine in regulating blood flow in the eye. *J. Ocular Pharmacol.* 4, 61–73.

Bruns, R. F., Lu, G. H. and Pugsley, T. A. (1986). Characterization of the A_2 adenosine receptor labeled by ^{3}H NECA in rat striatal membranes. *Mol. Pharmacol.* 29, 331–46.

Camras, C. B., Zhan, G.-L., Wang, Y. L., Toris, C. B. and Yablonski, M. E. (1994). Effects of (R)-phenylisopropyladenosine, an adenosine A-1 agonist, on aqueous humor dynamics in rabbits. *Invest. Ophthalmol. Vis. Sci.* 35 (ARVO abstracts), 2052.

Crosson, C. E. (1992). Ocular hypotensive activity of the adenosine agonist (R)-phenylisopropyladenosine in rabbits. *Curr. Eye Res.* 11, 453–8.

Crosson, C. E. (1995). Adenosine receptor activation modulates intraocular pressure in rabbits. *J. Pharmacol. Exp. Ther.* 273, 320–6.

Crosson, C. E. and Gray, T. (1994a). Adenosine A-1 and A-2 agonists modulate aqueous humor dynamics by separate mechanisms. *Invest. Ophthalmol. Vis. Sci.* 35 (ARVO abstracts), 2052.

Crosson, C. E. and Gray T. (1994b). Modulation of intraocular pressure by adenosine agonists. *J. Ocular Pharmacol.* 10, 379–83.

Crosson, C. E. and Gray, T. (1996). Characterization of ocular hypertension induced by adenosine agonists. *Invest. Ophthalmol. Vis. Sci.* 37, 1833–9.

Funk, R. H., Wagner, W. and Rohen, J. W. (1992). The effect of epinephrine on ciliary process vasculature and IOP studied by intraocular microendoscopy in the albino rabbit. *Curr. Eye Res.* 11, 161–73.

Gabelt, B. T., Robinson, J. C., Hubbard, W. C., Peterson, C. M., Debink, N., Wadhwa, A. and Kaufman, P. L. (1994). Apraclonidine and brimonidine effects on anterior ocular and cardiovascular physiology in normal and sympathectomized monkeys. *Exp. Eye Res.* 59, 633–44.

Gabelt, B. T., Robinson, J. C., Gange, S. J. and Kaufman, P. L. (1995). Superior cervical ganglionectomy in monkeys: Aqueous humor dynamics and their responses to drugs. *Exp. Eye Res.* 60, 575–84.

Hahnenberger, R. W. (1976). Influence of cataleptoid anaesthetic agents on the intraocular pressure in monkeys (*Macaca fascicularis*). *Acta Ophthalmol.* 54, 491–9.

Jones, R. F. and Maurice, D. M. (1966). New methods of measuring the rate of aqueous flow in man with fluorescein. *Exp. Eye Res.* 5, 208–20.

Kaufman, P. L. (1985). Aqueous humor dynamics. In (Ed. Duane, T. D.). Clinical ophthalmology. Vol. 3, chapt. 45, Pp. 1–24. Harper & Row: Philadelphia, U.S.A.

Kaufman, P. L. and Davis, G. E. (1980). Minified Goldmann applanating prism for tonometry in monkeys and humans. *Arch. Ophthalmol.* 98, 542–6.

Kaufman, P. L., True-Gabelt, B. and Erickson-Lamy, K. A. (1988). Time-dependence of perfusion outflow facility in the cynomolgus monkey. *Curr. Eye Res.* 7, 721–6.

Krootila, K., Oksala, O., Zschauer, A., Palkama, A. and Uusitalo, H. (1992). Inhibitory effect of methysergide on calcitonin gene-related peptide-induced vasodilation and ocular irritative changes in the rabbit. *Br. J. Pharmacol.* 106, 404–8.

Poyer, J. F., Gabelt, B. and Kaufman, P. L. (1992). The effect of topical $PGF_{2\alpha}$ on uveoscleral outflow and outflow facility in the rabbit eye. *Exp. Eye Res.* 54, 277–83.

Schiffmann, S. N. and Vanderhaeghen, J. J. (1993). Age-related loss of mRNA encoding adenosine A_2 receptor in the rat striatum. *Neurosci. Lett.* 158, 121–4.